A Comparative Study of Hormone Receptors in Spontaneously Developed, Steroid Hormone-Induced and Carcinogen-Induced Mammary Tumors in Female Noble Rats

Cheung Shu Yan

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Abstract

Breast cancer is the most prevalent endocrine-related malignancy in women. Several animal models are currently available for the study on this cancer. However, a proper evaluation on the hormone receptor status on these animal models is still unavailable.

In the present study, mammary tumors were induced in the adult female Noble (Nb) rats by a prolonged combined treatment of estrogen and androgen or by a single dosage of a carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA). Spontaneously developed mammary tumors were also developed in high incidence rate in the aged female Nb rats. The incidence rates, lengths of latency period and histopathology of the mammary tumors developed in these three different rat models were compared. In order to investigate the involvement of different hormones in the mammary gland carcinogenesis, the expression patterns of estrogen receptor (ERα & ERβ), progesterone receptor (PR), androgen receptor (AR) and prolactin receptor (PRLR) in these spontaneously developed, hormone-induced and carcinogen-induced breast tumors were also characterized and compared by immunohistochemistry and western blotting.

The results of immunohistochemistry showed that mammary glands in the normal female Nb expressed ERα, ERβ, PR, AR and PRLR. Moderate to strong immunoreactivities of ERα, ERβ, PR, AR and PRLR were also detected in the spontaneously developed and the hormone-induced mammary tumors. However, the carcinogen-induced carcinomas exhibited a different hormone receptor patterns. Moderate to strong immunoreactivities of ERα, PR, PRLR and AR were detected in the mammary tumors. These carcinogen-induced tumors expressed a negative to weak ERβ immunoreactivity.

The expressions of the hormone receptors in the mammary tumors of these three different models were further investigated by western blotting. It was observed that an

expression of the native 67kDa ER protein was detected in the normal mammary glands as well as the spontaneously developed, hormone-induced and carcinogen-induced mammary tumors. On the other hand, the DMBA-induced neoplasms exhibited a weaker expression of this native 67kDa ERα protein. Besides, some of the DMBA-induced tumor samples also exhibited a weak expression of the putative 50kDa ERα isoforms. Moreover, the putative 50 and 54 kDa ERα isoforms were also detected in the spontaneously developed mammary tumors. On the other hand, there were expressions of PR isoform, PR-C, in the normal mammary glands, spontaneously developed, carcinogen-induced and T+E_2-induced mammary tumors. However, in the T+DES induced carcinomas, protein expression of PR-C was not detected. It was also observed that mammary glands and all classes of mammary tumors induced in female Noble rats express PR-A2 isoforms. Elevated expressions of PR-B and PR-A1 isoforms were also detected in the spontaneously developed carcinoma. Furthermore, an elevated expression of AR isoform, AR-B, was detected in all classes of mammary tumors as compared with the normal gland. Expressions of AR-A isoform were also detected in the spontaneously developed mammary tumors. Finally, western blot analysis for PRLR has detected expressions of the long PRLR isoform in both normal and neoplastic mammary samples.

These observations demonstrate the notion that the process of mammary gland carcinogenesis in the DMBA-induced model may be different from that of the spontaneously developed and hormone-induced models in the female Noble rats. As mutations of different oncogenes and tumor suppressor genes are involved during the mammary gland carcinogenesis in these three different animal models, the conversion of the varied genetic information into a defined phenotype may lead to a discrepancy in the hormone receptor status between the carcinogen-induced mammary tumors and the spontaneously developed as well as the hormone-induced neoplasms.

擇要

乳腺癌是女性中發病率最高的致命疾病，目前有數種動物模型可供研究人類乳腺癌之用。本實驗中，我們分別利用了類固醇激素以及化學致癌物質，在一鼠類物種中建立了兩類乳腺癌的動物模型。另一方面，我們從該鼠類物種中，亦採集了一批自發的乳腺腫瘤。

我們比較了三種動物模型中乳腺腫瘤的發病率、潛伏期以及組織病理學。我們亦運用了免疫組織化學法以及西方蛋白印迹法，比較了激素受體在三種動物模型中的表達模式。這些激素受體包括了雌激素受體、雄激素受體、孕酮受體和催乳激素受體。是次研究中，我們發現類固醇激素能夠加速鼠類自發乳腺腫瘤的形成。除此之外，我們亦發現激素受體在化學致癌物質誘導的動物模型中整體的表達模式，有別於類固醇激素誘導以及自發的乳腺癌動物模型。我們相信這些乳腺腫瘤表型上的差異，是基於化學致癌物質誘導的動物模型具有獨特的致癌機制。我們相信是次研究，對動物模型日後在人類乳腺癌研究上的運用，具有深遠的啟發作用。

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Contents

Chapter 1. Introduction

1.1 Epidemiology of Breast Cancer

1.1.1 Epidemiology of Breast Cancer in Females

Breast cancer is the most prevalent malignancy among women, with about 1 million new cases in the world every year (McPherson *et al.*, 2000). This disease accounts for 18 % of all female cancers in the world (Table 1.1). Obviously, breast cancer has become a serious worldwide health problem among women. The emotional, social and healthcare costs related to this cancer are enormous. Therefore, breast cancer is such a matter that worthy of our full concerns.

Although breast cancer shows an overall high incidence in the world, its incidence rate varies markedly among countries. The incidence of female breast cancer is highest in the United States and Northern Europe, intermediate in the Southern and Eastern Europe as well as the South America, but lowest in Asia (Parkin *et al.*, 1992). Figure 1.1 shows the international variation in the breast cancer incidence among women from 1988 to 1993.

In the United States, where the highest incidence rate is reported, approximately 180,000 women are diagnosed with breast cancer annually (Kelsey and Horn-Ross, 1993). This incidence rate predicts that 1 in 8 American women will develop breast cancer during her lifetime and approximately 30 % of these women will die of this disease (Ries *et al.*, 1998). Currently, breast cancer is the second leading cause of cancer deaths among American women after lung cancer and is the leading cause of death in the age group between 50 and 55 (Parker *et al.*, 1997). Although its number is already high, adding to the concern is the fact that breast cancer has been rising steadily in all age groups since 1930 (White *et al.*, 1990; Parker *et al.*, 1997).

Apart from the United States, a nearly doubled sharp increase in the breast cancer incidence is also reported recently in the traditionally low-risk Asian countries such as Japan, Singapore and the urban areas of China since 1950s (Jin *et al.*, 1993; Seow *et al.*, 1996; Nagata *et al.*, 1997).

Although this worldwide rise in breast cancer incidence may be partially attributable to the increased use of screening mammography and the aging of the population (Chu *et al.*, 1996), it is undeniable that the increasing number reflects a real trend, suggesting that environment or lifestyle changes may be effecting an increase in the cancer incidence (Brinton and Schairer *et al.*, 1993; Malone *et al.*, 1993; Garfinkel *et al.*, 1994).

Although the incidence of breast cancer shows a global rise, its mortality levels off or presents a slight decline in most Western countries since 1990. Generally speaking, countries which show a recent downturn in death rate are those with the highest incidence and mortality, such as the United Kingdom, Netherlands, Sweden and the United States. The countries demonstrating increasing death rates tend to be those with the lowest mortality. For instance, Poland and Spain, which have the lowest breast cancer mortality among European countries, show a rising death rate (Beral *et al.*, 1995; Hermon *et al.*, 1996).

1.1.2 Incidence and Morality of Female Breast Cancer in Hong Kong

According to a recent study conducted by the Hong Kong Cancer Registry, our society has the highest breast cancer incidence in the Asia, with 1,500 women diagnosed annually. Among the local population, it is estimated that one out of every forty women will develop breast cancer during her lifetime.

Over the past 20 years, the incidence of female breasts cancer has been increasing obviously, especially in the age group between 40 and 49 (Figure 1.2 &

1.3). The causes for this dramatic rise are uncertain but are believed to be attributable to the increasing westernized dietary habits and lifestyle among the population since the Second World War. On the other hand, the local death rate due to breast cancer has also increased three-fold in the last 20 years. In 1998, it is found that 380 women died of this disease, as compared with only 111 sufferers in 1961. Due to this dramatic increase rate, breast cancer has overtaken lung cancer as the leading cancer among the local females since 1994 (Figure 1.2).

1.1.3 Epidemiology of Breast Cancer in Males

In contrast to its prevalence among the women, breast cancer only rarely occurs among males in all parts of the world. In the United States, it was reported that only approximately 1,600 new cases of male breast cancer were diagnosed and 400 deaths occurred as a result of this cancer in 1998. Epidemiological statistics further showed that male breast cancer only accounts for approximately 1 % of all breast cancers and less than 1 % of all annual cancer deaths in the America males (Landis *et al.,* 1998). In other populations, the incidence rate of male breast cancer is only on the order of 1 case per 100,000 men annually or even less. The highest incidence rate of breast cancer is found in Brazil, where an incidence rate of 3.4 cases per 100,000 males is recorded. In other Asia countries like Singapore and Japan, an even lower incidence rate of 0.1 cases per 100,000 is found (Muir *et al.,* 1987).

Previous studies suggest that some cases of male breast cancer may be caused by pathological conditions that result in relative estrogen excess or lack of androgen (Thomas, 1993). The strongest risk factor for developing male breast cancer is the Klinefelter's syndrome. It is a rare condition that results from the inheritance of an additional X chromosome (Hultborn *et al.,* 1997). Men with this condition have

atrophic testes, gynecomastia, high levels of gonadrotropins and low plasma levels of testosterone. This results in high estrogen-testosterone ratio. Men usually need to expose to this aberrant hormonal milieu for decades before breast cancer arises. The risk of male breast cancer in these individuals is up to 50 times higher than for men with a normal genotype (Thomas, 1993).

As male breast cancer only represents rare cases, in the following sections, emphasis will only be put on the female breast cancers.

1.2 Risk Factors for Female Breast Cancer

Breast cancer is a complex and multi-staged disease which involves an intense interaction between the inherited and the environmental factors. Some of the known and established risk factors for breast cancer are categorized in Table 1.2 and discussed as follows:

1.2.1 Genetic Risk Factors

Family history is the most significant known risk factor for the incidence of breast cancer. Epidemiological studies show that among all the female breast cancer patients, there are about 15 to 20 % of the sufferers have a family history (Slattery and Kerber, 1993). If early-onset and bilateral breast cancer is found in a woman's first-degree relative, which includes the individual's mother, sisters or daughters, she will have a high risk to develop the disease (McPherson *et al.*, 2000).

The susceptibility for breast cancer is generally inherited as an autosomal dominance with limited penetrance. It can be transmitted through either gender, with some family members possessing the abnormal gene without developing the cancer themselves (McPherson, *et al.*, 2000). Two major breast cancer susceptibility genes, BRCA1 and BRCA2, have been identified and they approximately account for 15 to

20 % of familial breast cancer (Slattery and Kerber, 1993). Other heritable breast cancer factors are underway to be identified.

The first breast cancer susceptibility gene BRCA1 was isolated in 1994 (Miki *et al.*, 1994). This breast cancer gene is located on the long arms of chromosome 17. The biological function of the BRCA1 is still unclear and controversial. However, there are accumulated evidences showing that BRCA1 plays roles in the gene transcription, control of cell cycle as well as the DNA repair pathways (Martin and Weber, 2000). BRCA1 is a large gene. Germline mutation of BRCA1 can occur nearly in the entire sequence, thereby enhancing the occurrence of a variety of mutations within this gene. In general, female with BRCA1 mutation have a 60-80 % lifetime risk for developing breast cancer and a 20-40 % risk for ovarian cancer (Struewing *et al.*, 1996). Breast caners with BRCA1 mutations demonstrate a number of distinct clinical features including onset in earlier age as compared with the sporadic cases, a higher frequency of bilateral breast cancer, and occasionally the presence of associated tumors, specifically ovarian cancer and possibly colon and prostate cancer (Nelson *et al.*, 1993).

The second breast cancer susceptibility gene BRCA2 was identified in late 1995 and is localized to the long arms of chromosome 13, (Wooster *et al.*, 1995). Similar to that of BRCA1, the function of BRCA2 is believed to be related to the DNA damage response pathways (Martin and Weber, 2000). BRCA2 is one of the largest genes isolated so far and is approximately twice as large as BRCA1. Although the mutations of BRCA2 are not well studied as that of BRCA1, there are more than 250 known mutations spreading throughout the gene have been found. The BRCA2-mutation sufferers have cancer risk profile similar, but not identical, to that of BRCA1 mutation victims. Lifetime risk for breast cancer in the BRCA2-

mutation victims is about 85 % whereas it is in the range of 10 to 20 % for the ovarian cancer risk which is higher than that associated with the BRCA1 mutations (Ford *et al.,* 1998). Contrary to the male BRCA1 mutation carriers, men with BRCA2 mutations are associated with a 6 % lifetime breast cancer risk, representing a 100-fold increase over the general population. BRCA2 mutations are also linked to an increasing incidence in the colon, prostate, pancreatic, gallbladder, bile duct, and stomach cancers as well as the malignant melanoma (Cancer Research Campaign Genetic Epidemiology Unit, 1999).

1.2.2 Hormonal Risk Factors

Breast cancer is an endocrine-related cancer. The breast carcinogensis and the subsequent tumor progression are directly associated with exposure to hormones, particularly the estrogen, which may contribute to the mammary gland carcinogenesis by increasing the cell division and rate of cell proliferation, thereby enhancing the accumulation of random genetic errors (Preston-Martin *et al.,* 1991). It is generally believed that prolonged or increased exposure to either endogenous or exogenous estrogens will result in an increased breast cancer risk (Persson, 2000; Clemons and Goss, 2001). Therefore, the hormone-related factors including early menarche, late menopause and nulliparity, that will increase the number of menstrual cycles and subsequently enhance the endogenous estrogen exposure, are able to increase the breast cancer risk. Similarly, factors that are related to the supplement of exogenous estrogen will also increase one's likelihood for breast cancer development. Examples are administration of oral contraceptives and estrogen replacement therapy for the menopausal women. On the other hand, reduction in the total number of ovulatory cycles, which can be achieved by moderate level of exercise and longer lactation period, are found to be protective against breast cancer

incidence (Martin and Weber, 2000). These endogenous and exogenous breast cancer risk factors are discussed individually in the following.

1.2.2.1 Endogenous Hormonal Risk Factors

Epidemiological studies have showed that women who start menstruation early in life and have a late menopause will suffer from an increased risk of developing breast cancer. This clearly demonstrates that the breast cancer is related to the ovarian steroid hormones. Solid evidences are illustrated by the fact that women who start menarche at age 12 or earlier with rapid establishment of regular cycles have an almost fourfold risk on breast cancer, as compared with women who menstruate at age 13 or older with longer duration of irregular cycles (Henderson *et al.,* 1985). On the other hand, females who have natural menopause after age 55 are twice more likely to develop breast cancer than individuals who experience the menopause before the age of 45 (McPherson and Dixon, 2000).

The geographic variations in the breast cancer prevalence may also be explained by the differences in the menarcheal and menopausal ages. In Asia, where a lower breast cancer incidence is observed, earlier menacheal and later menopausal ages are reported, as compared with the corresponding data in the United States, which shows the largest breast cancer numbers.

Pregnancy is the other endogenous hormonal factor related to the breast cancer risk. Both nulliparity and late first birth are reported to be able to raise one's lifetime breast cancer susceptibility (Kelsey and Gammon, 1993). The highest risk is found in females who give their first birth after the age of 35. These individuals also show a higher breast cancer frequency than the nulliparous women. In contrast, early full term pregnancy is protective against this cancer. Women with early second birth can further reduce the breast cancer risk (McPherson and Dixon, 2000). The

mechanism by which full-term pregnancy alters subsequent breast cancer risk is unknown, although it is generally believed that it may due to the priming effect brought by the terminal differentiation of breast epithelium during the pregnancy (Martin and Weber, 2000).

Finally, body weight is also a suspected risk factor. It is found that obesity can result in twofold increase in the breast cancer risk in postmenopausal women (McPherson and Dixon, 2000). This observation in postmenopausal women may be attributed to the fact that the majority of endogenous estrogen comes from the conversion of androstenedione to estrone in the adipose tissue, and thus, obesity will result in the long-term increase in estrogen exposure.

1.2.2.2 Exogenous Hormonal Risk Factors

Due to the close association between the endogenous hormone secretions and the breast cancer risks, the potential influences of exogenous estrogen have evoked widespread attentions.

Since the introduction of oral contraceptives for birth control in 1960s, there have been many studies to analyze its possible influence on the breast cancer risk. However, the results are inconsistent and such controversial results may be due to the temporal changes in hormone dosages as well as the drug delivery methods (Fraser, 2000). Overall, it is now believed that oral contraceptive will only cast a small risk on the users. Nevertheless, females who are currently using the oral contraceptives and are in extended use will have higher breast cancer risk than the general population (Martin and Weber, 2000).

On the other hand, hormone replacement therapy, which is mainly adopted by the postmenopausal women for the relief of menopausaul symptoms, reduction in cardiovascular disease and delay in osteoporotic complications, has also been under

extensive studies (Torgerson, 2000). Short-term uses of estrogen replacement are shown to be safe but the risk on breast cancer development will increase annually after continuous usage for 5 years. Although the use of hormone replacement therapy is beneficial to postmenopausal women, it is still a possible risk for the women, especially for the long-term users and individuals with family history on breast cancer or personal history on dysplastic breast disease. Regular check-up for the breast and endometrial cancer is recommended for those women with hormone replacement therapy (Purdie, 2000).

The synthetic estrogen, diethylstilbestrol (DES), was widely prescribed to the pregnant females in order to minimize pregnancy complications and prevent abortions between 1943 and 1971. However, subsequent studies have indicated that administration of DES during the period of mammary gland proliferation, such as the pregnancy, can significantly increase one's breast cancer risk. This drug has also been found to cause other serious physical and psychological damage to the pregnant users and to their offspring. This drug is now no longer prescribed (Saunders, 1988).

1.2.3 Other Risk Factors

Some examples of the risk factors for breast cancer have been reported. These include personal history such as increasing age, previous history of hyperplastic disease or cancer history, radiation exposure as well as lifestyle such as high-fat diet, excess alcohol intake and smoking (Martin and Weber, 2000; McPherson and Dixon, 2000). Among these factors, age is the most significant environmental risk factor. It is estimated that the susceptibility on breast cancer will double every decade until menopause in women, after which a sharp reduction in the increase rate is observed. This positive relation between age and breast cancer incidence is attributable to the lifetime accumulation of carcinogenic mutations

whereas the dramatic decline in breast cancer risk after menopause is explained by the reduction in endogenous estrogen production (McPherson and Dixon, 2000).

1.3 Oncogenetic Basis of Female Breast Cancer

Current views on the oncogenetic basis of female breast cancer suggest that the malignant transformation of the normal breast epithelium into an invasive carcinoma is a multistep process, in which a number of genetic alternations have occurred within various critical genes, that are directly or indirectly responsible for regulating certain important cellular processes, such as cell proliferation, differentiation, chromosomal replication and apoptosis (Lakhani, 1999). These genetic alternations can be induced by either the internal or external causes. The internal causes, which all arise from the body itself, include those risk factors such as early menarche, late menopause, nulliparity, aging, obesity and the inheritance of mutated BRCA1 or BRCA2 gene. On the other hand, the external causes are environmental factors such as the administration of oral contraceptives, hormone replacement therapy, radiation exposure, high-fat diet, excess alcohol intake and smoking. Overall, no single factor can fully explain the incidence of breast cancer. Instead, breast cancer is a complex disease, of which the development involves interplay of a number of etiologic agents, and they arise either internally or externally (Weinstein, 1988). These internal or external etiologic agents can either activate or inactive the affected genes. For a cancer to develop, oncogenes should be activated whereas tumor suppressor gene should be inactivated (Weinberg, 1989).

Oncogenes are a class of genes that can either stimulate cell division or prevent cell death, thereby possessing the ability to cause tumor growth and development if these genes are activated. In humans, oncogenes are rarely activated

by viruses, such as the mouse mammary tumor virus (MMTV) in murine. Instead, activation of oncogene is commonly triggered by one of three major mechanisms, mainly by amplification of genes, occasionally by alterations of amino acids and chromosomal translocation. In the amplification mechanism, multiple copies of genes are reproduced in a single chromosome. By increasing the gene dosage, the corresponding protein products are over-expressed, thereby facilitating distorted cellular development. Examples of the breast caner related oncogenes activated by this amplification mechanism include epidermal growth factor receptor (EGFR), erbB-2/HER-2/neu, c-myc and cyclin D_1, all of which can stimulate cellular divisions (Devilee *et al.*, 1994).

Tumor suppressor genes are a class of genes that can inhibit tumor growth when in normal functioning. For a tumor to develop, both of the functional tumor suppressor alleles on a single chromosome must be inactivated by mutations, which occur mainly sporadically rather than by inheritance. In breast cancer, the most commonly mutated tumor suppressor gene is p53, which is a checkpoint regulator in the cell cycle and it monitors the fidelity of mitosis. In the absence of p53, chromosomes segregate aberrantly and cells soon become polyploid. Inherited mutation of p53 is seldom a sole cause of breast cancer. Instead, p53 principally alters by sporadic somatic mutation. It is found that nearly half of all invasive breast tumors will carry a mutated p53 (Devilee et al., 1994). In comparison, mutation of the tumor suppressors BRCA1 and BRCA2, which are involved less than 10 % of all breast cancer case, are mainly inherited and thus, seldom found in the sporadic breast cancer (Rajan et *al*, 1996). On the other hand, some tumor suppressors such as Rb and p16, which are parts of the EGF responsive pathway and associated with stimulating cell division, are rarely mutated in breast cancer.

Currently, our understanding on the genetic basis of breast cancer is still incomplete, extensive studies are now underway to identify more breast cancer related oncogenes and tumor suppressors genes.

1.4 Hormonal Basis of Female Breast Cancer

The mammary gland is a target organ by a number of ovarian and pituitary hormones. The normal development of the breast as well as the pathological progression of the breast tumors are under strict control and influence by an extensive interplay between hormones and growth factors, which are secreted by the hypothalamus, pituitary, adrenal gland, thyroid gland and particularly the ovary (Russo and Russo, 1998). Of all the hormones involved, estrogen, progesterone and prolactin are considered in great significance, but the role of androgen is still ambiguous. The following sections give a detailed review on these hormones and their role in mammary gland carcinogenesis.

1.4.1 Mechanisms of Hormone Action

1.4.1.1 Estrogen and Progesterone

The ovarian steroid hormones, estrogen and progesterone, are well-established tropic factors that promote proliferation and differentiation of the breast epithelial cells. The expression and presence of both the estrogen and progesterone receptors in the breast epithelium gives strong support that the action of these two steroids in the mammary gland is mainly mediated by binding to their nuclear receptors (Figure 1.3). The activated hormone-receptor complex then migrates to the nucleus, complements to the target hormone-responsive genes and finally modulates the transcription of genes. (Russo *et al,* 1999; Hansen and Bissell, 2000).

Estrogen receptor (ER) exists in two isoforms, ERα and ERβ, each being encoded by separate genes (Girdler and Brotherick, 2000). In the normal mammary glands and some breast tumors, there is a co-expression of ERα and ERβ. However, it is still uncertain whether these two isoforms have the same or different physiological roles in the normal and neoplastic mammary tissues (Osborne, 1998; Nass and Dividson, 1999).

On the other hand, progesterone receptor (PR) also exists in two isoforms, namely PR-A and PR-B. Unlike the ER subtypes, PR isoforms are transcribed from two distinct transcription start sites within the same gene (Clemm, 2000; Hansen and Bissell, 2000). These two receptor isoforms show differences in the transcriptional activation properties and have distinct expression ratios in various target organs. For instance, PR-A isoform is predominantly expressed in the rodent mammary gland, in a ratio of 3:1 in comparison with the PR-B. The appropriate cellular responsiveness to progesterone is believed to be dependent on the regulated hormonal activity as well as the accurate PR isoform expression ratio. Imbalanced PR expression may cause inappropriate progesterone signaling which will result in aberrant mammary development (Shyamala *et al.,* 1999). Additional pieces of evidence shows that progesterone signaling may also be affected by the presence of a third PR, PR-C, a protein missing the first 594 amino acids at the common N-terminus of PR-A and PR-B. At present, the role of PR-C is still not well defined (Hansen and Bissell, 2000).

Finally, other ER and PR variants and mutants are also present and may contribute to the hormonal sensitivity and development of the breast tumors. However, their roles in mammary gland carcinogenesis are still not defined yet.

More studies are required to define their roles (Osborne, 1998; Hansen and Bissell, 2000).

In the mammary gland, estrogen and progesterone are mutually regulating each other's activities. In female breast, the expression and synthesis of PR is under positive estrogenic regulation. On the other hand, progesterone antagonizes estrogen action by decreasing the replenishment of ER and promoting the synthesis of 17β-hydroxysteroid dehydrogenase which results in the accelerated metabolism of estrogen to the inactive estrone form (Shyamala *et al.*, 1999).

1.4.1.2 Prolactin

Prolactin also mediates its function through receptor binding (Figure 1.4). After binding to its specific cell membrane-spanning receptor, the peptide hormone activates subsequent signaling pathway, leading to the transcription of target genes, which may induce mammary proliferation, differentiation as well as the lactogenesis (Hennighausen *et al.*, 1997).

Prolactin receptor (PRLR) principally exists in two isoforms, the long and the short PRLR, which are generated by alternative splicing from a single gene and only differs in the length of their cytoplasmic domains. Long PRLR is dominantly expressed in the breast whereas its short form counterpart is mainly found in the liver (Shirota *et al.*, 1990). The third intermediate form, the Nb2 form, is a deletion mutation in the cytoplasmic domain of the long PRLR. Both the long and short PRLR present in the normal and malignant mammary cells. In some breast tumors, expression of the Nb2 from is also detected (Hennighausen *et al.*, 1997).

1.4.2 Hormonal Regulation of Normal Breast Development

The normal breast development is divided into several stages. At birth, the mammary gland only consists of few primary ducts embedded the mammary fat pad with seldom branching. Between birth and puberty, the growth of the mammary tissues is mainly hormone-independent and there is primarily an increase in amount of stromal tissue. During this prepubertal period, estrogens in the developing females will interact with the primary epithelial-stromal unit to promote mammary ductal development and enhance its penetration into the fatty tissue. In contrast, androgen inside the developing male interacts with the epithelial-stromal unit to induce destruction of the under-developed mammary epithelium (Nass and Davidson, 1999).

Following the priming effects brought by estrogens, the mammary gland of the prepubertal females remains quiescent until the establishment of the full menstrual cycles during puberty. Estrogen, which is secreted at maximal level by the ovaries during ovulation, brings about an extensive mammary ductal proliferation. On the other hand, the ovulatory progesterone is responsible for the lobuloalveolar development by enhancing mammary cell differentiation. Prolactin is mainly synthesized by the anterior pituitary and to a lesser extent produced locally by the breast (Binart *et al.*, 2000). Prolactin is responsible for the maturation of the primary mammary ductal system. It promotes lobuloalveolar branching from the terminal end buds. The terminal end buds will regress after the completion of its differentiation into alveolar buds and lobules (Horseman, 1999).

Estrogen plus progesterone, prolactin and other hormonal factors, when secreted in an adequate balance, give a complete and proper development of the mammary gland. The full mammary maturation is characterized by the regression of terminal end buds with the presence of highly branched lobular ducts (Horseman,

1999). After sexual maturity, the development of mammary glands stops fundamentally, although the epithelial tissue shows alternating rounds of proliferation and apoptosis in responsive to the cyclic hormonal stimuli by the ovarian steroids during menstrual cycle. The greatest increase in mitotic index of the mammary gland occurs during the luteal phase. This suggests that progesterone is responsible to this mammary periodic change (Nass and Davidson, 1999).

During pregnancy, prolactin, progesterone, estrogen and other endocrine factors like placental lactogens stimulate further lobuloalveolar differentiations. After this physiological lobuloalveolar development has been completed, the mammary gland becomes competent to carry out lactation. The delivery of prolactin and other factors such as growth hormone (Feldman *et al.*, 1993), in the context of falling progesterone secretions, stimulates gene expressions of various milk proteins and subsequently leads to the lactogenesis (Horseman, 1999).

After lactation, there is a decline in hormone secretions and it initiates the involution of mammary gland. During the weaning period, the basement membrane of glandular alveoli breaks down and extensive cellular apoptosis occurs. After involution has been completed, the mammary gland only composes of a highly branched ductal system with some remaining alveoli (Nass and Davidson, 1999)

1.4.3 Hormonal Regulation of Breast Carcinogenesis and Its Subsequent Progression

Breast cancer is a multi-stepped and hormone-related disease. The initial malignant transformation and the subsequent cancer progressing processes, such as

invasion, migration, angiogenesis and evasion of immune system, are largely dependent on hormonal regulation, which may act in the endocrine, paracrine or autocrine manners (Nass and Davidson, 1999). The ovarian steroids, estrogen and progesterone, as well as the pituitary prolactin are the active participants in these processes. On the other hand, androgen may also play a role in the breast cancer development. The roles of different hormones on the mammary carcinogenesis are reviewed as follows.

1.4.3.1 Androgen

Although there is widespread co-expression of androgen receptor (AR) with ER and PR in human breast tumors, the role of androgen and its receptor in female breast cancer is still controversial as androgen is described having both the cancer growth stimulatory and inhibitory effects in human breast cancer cell lines. The mechanisms by which androgens exert these conflicting effects in cancer cell lines and also in breast cancer tissues are still unknown. To determine the role of the AR-mediated pathways in regulating breast tumor growth, further studies are warranted (Birrell *et al.*, 1998; Bry&sacute, 2000).

Recently, additional pieces of evidence are available from the animal studies and the experimental results indicate that androgen might play a role in the mammary carcinogenesis. In both the male and female Noble (Nb) rats, it has been found that a combined androgen and estrogen prolonged treatment was able to induce high incidence of mammary tumors within relatively short time. The supplement of androgen was found to be able to shorten the tumor latency period, as compared with the corresponding data on the estrogen-induced mammary tumors in Nb rats (Liao *et al.*, 1998; Xie *et al.*, 1999; 1999b).

Overall, the action of androgen on breast cancer is speculated to be multi-discipline. On one hand, androgen promotes the proliferation of breast cancer cells directly via the androgen receptor-mediated mechanism or by its stimulation on the synthesis of other growth factors. On the other hand, the action of androgen may be indirect. Firstly, androgen can be converted into estrogen by the enzyme aromatase, (Bernstein and Ross, 1993). Estrogen, derived from the systemic conversion in peripheral tissues or from the local conversion in the breast tumors, participates in the maintenance and growth of the breast tumors in the carriers, especially in the postmenopausal females (Chen, 1998). Alternatively, androgen acts indirectly by increasing the circulating amount of free estradiol, via either reducing the secretions of hepatic estradiol-bound sex hormone binding globulin (SHBG) by the liver or decreasing the fraction of the SHBG, thereby providing substantial amount of estrogen for tumor growth (Lonning *et al.*, 1995).

1.4.3.2 Estrogen

The most well-documented risk factors of breast cancer, such as early menarche, late menopause and nulliparity, are associated with the most significant physiological changes in the estrogen secretion during a female's lifetime. This strongly suggests that estrogen plays a very important role in the development of the human breast cancer.

Clinical studies have found that about two thirds of the primary breast tumors are ERα-positive. In comparison with the ERα negative tumors, neoplastic tissue expressing ERα are generally more well-differentiated, grow more slowly and are associated with longer disease-free survival (Osborne, 1998). Expression of ERα in breast cancer also shows important significance for prognosis, as generally ER-

positive tumors are more responsive to endocrine therapy with antiestrogens such as tamoxifen. Although multiple lines of evidence also show that ERβ is also expressed in both the normal and malignant breast cells, the contribution of this ERβ expression to the development of normal and tumorigenic breast cells is still completely undefined at present (Osborne, 1998; Girdler and Brotherick, 2000).

In the functional studies, estrogen was found to be able to stimulate cell cycle progression in the ERα expressing cancer cells, which results in the proliferation of breast tumors. In addition to its role as a mitogen, estrogen also acts as a survival factor for the ER-positive tumor cells as estrogen ablation is commonly followed by induction of apoptosis. It is believed that estrogen maintains the tumor survival by modulating the expression of an antiapoptotic protein, Bcl-2. This belief is based on the observation that the increases in Bcl-2 expression in breast cancer cells can be significantly inhibited by the administration of anti-estrogens (Nass and Davidson, 1999).

Other studies have demonstrated that when the ERα positive breast tumors continue to develop, estrogen may finally lose its influence and the tumors become no more responsive to either estrogen or tamoxifen. Several possible mechanisms are proposed to explain this progression of hormone-independence. These include the ligand-independent ER activation, the expression of variant or mutant ER forms as well as the altered expression of the downstream estrogen targets (Ferguson and Davidson, 1997). Furthermore, it is observed that when the ERα positive primary breast tumors have invaded to a secondary site, up to 30 % to 40 % of this metastases will become ERα negative (Kuukasjarvi *et al.*, 1997). As estrogen is responsible for promoting growth and survival of ERα positive cells, loss of ER expression may be

an important step in the progression of breast tumors into a more aggressive form, and this represents that the cancer cells have acquired the ability to bypass the ERα pathways for growth and survival. In these ERα negative tumors, inhibition of ER gene transcription is a likely mechanism responsible for the loss of ERα expression. This inhibition may be achieved by the methylation of cytosine-rich areas, termed CpG islands, in the 5' regulatory region of the ERα gene (Lapidus *et al.*, 1996).

1.4.3.3 Progesterone

Besides estrogen, progesterone can also enhance the proliferation of breast cancer cells. Studies using agents targeting the activity of progesterone receptor have shown that these agents can alter subsequent tumor growth. Administration of antiprogestins and pharmacological dosage of progesterone can generally result in the inhibition of cancer growth. A mechanism has been proposed recently to account for this mitogenic action by progesterone. This hypothesis is based on the observation that during the luteal phase of menstrual cycles, the elevated secretion of progestine is generally accompanied with a rise in the growth hormone production by the hyperplastic mammary epithelium as well as an elevated growth hormone level in the serum. This elevated local expression of mammary growth hormone can be blocked significantly by antiprogestins. Therefore, it is proposed that this progesterone-induced systemic and local growth hormone synthesis, instead of progesterone itself, are responsible for the cyclic breast cell growth during the luteal phrase and the proliferation of the breast cancer cells (Mol *et al.*, 1996).

Currently, it is still uncertain whether progesterone is a survival factors for the breast cancer cells or not. However, in the normal mammary gland, it is reported that progesterone can inhibit apoptosis during involution (Feng *et al.*, 1995). Apart

from the ERα, the PR expression is also an important prognostic indicator for breast cancer. PR is found co-expressed in about 50 % of all ERα positive breast tumors. This reflects that ERα is a key transcriptional factor for the expression of PR (Read *et al.,* 1988). The absence of PR expression in ERα positive tumors may imply nonfunctional or aberrantly functioning ERα and the neoplastic cells are no longer influenced by the administration of anti-estrogens. This theory is consistent with the clinical findings that double-positive tumors usually are responsive and amenable to endocrine therapy, in the frequency of about 75 %, whereas in the ERα-positive/PR-negative tumors, the frequency drops dramatically and only less than one third initially responds, and nearly all ERα-negative/PR-negative tumors rarely respond to tamoxifen (Nass and Davidson, 1999). It is widely believed that the absence of PR expression is also associated with the methylation in the regulatory region of the PR gene (Lapidus *et al.*, 1996).

In most breast tumors, it is found that PR-A and PR-B were expressed in similar amounts. The remaining proportion of breast tumors usually expresses a very low level of PR-B. However, the clinical significance of this expression pattern of PR-A and PR-B has not been addressed yet (Graham *et al.,* 1996).

1.4.3.4 Prolactin

Prolactin is well-known for its active involvement in the growth and differentiation of the normal mammary gland as well as its role in the initiation and maintenance of lactation (Vonderhaar, 1999). The role of prolactin in mammary cancer has been clearly demonstrated in rodents. Prolactin is found directly

contributing to the etiology of both spontaneously developed and carcinogen-induced murine mammary carcinoma (Welsch and Nagasawa, 1977). Prolactin also acts synergistically with ovarian steroids to promote the growth of human breast cancer xenografts in mice (Leung and Shiu, 1981). In rats, there is a direct association between the serum prolactin level and the susceptibility of different rat strains to the chemical carcinogen induction of mammary tumors (Boyns *et al.*, 1973). Tumors induced in rat by carcinogens, nitrosomethylurea (NMU) or 7,12-Dimethyl-benz[a]-anthracene (DMBA) are dependent on prolactin for sustained growth (Mershon *et al.*, 1995). Prolactin is also a mitogen for human breast cancer cells in culture, and anti-prolactin reagents can inhibit the growth of these cells (Ginsberg amd Vonderhaar, 1995).

In contrast to this clear demonstration in the rodent models, the role of prolactin in human breast tumorigenesis is poorly defined because there is a lack of correlation between circulating prolactin levels and the incidence of breast tumors, and treatments that suppress pituitary prolactin release have not been shown to improve clinical outcome.

Nevertheless, a breakthrough is achieved recently. It is now known that prolactin can also be produced in many extrapituitary locations, including both the normal and the malignant breast epithelial cells (Vonderhaar, 1999). On the other hand, it is also found that prolactin receptors, both long and short form, are widely expressed in the normal glands and the mammary cancers. These findings lead to the hypothesis that locally produced prolactin in the breast tumors act as an autocrine or paracrine factor to support neoplastic growth, which is independent of the circulating prolactin levels (Reynolds *et al.*, 1997; Vonderhaar, 1999). Besides, there are also evidences showing that proalctin may also function synergically with estrogen and

progesterone to regulate the growth of normal and malignant breast tissues. In one study in a large panel of human breast cancer cell lines and primary tumors using quantitative Northern blot analysis, there shows a positive correlation between the expression levels of PRLR and that of ER and PR. In these breast cancer cell lines, treatment with progestins and estrogen was found to be able to increase PRLR expression while addition of exogenous prolactin resulted in elevated PR levels (Ormandy *et al.*, 1997).

Furthermore, growth hormone and prolactin are closely related pituitary hormones. Human growth hormone is found to be able to bind to and activate both growth hormone receptor (GHR) and PRLR. It is proposed that there is a cross-regulation between growth hormone and prolactin in the mammary carcinogenesis (Wennbo and Tornell, 2000).

1.5 Animal Models for Breast Cancer

Breast cancer is a very complex disease. Until now, our understanding on this disease is still incomplete and we still lack effective strategies for the prevention and cure on the breast cancer. In order to have a complete picture on the pathogenesis of this cancer, experimental animal models that highly mimic the human breast cancer are certainly needed. Breast cancer is comprised of a heterogeneous group of diseases, characterized by different sets of genetic mutations, histopathological types and metastatic potentials. Even in the same primary tumour mass, the neoplastic mammary cells would show heterogeneous phenotypic characteristics. Therefore, it is unlikely that any single animal models will be able to mimic all the aspects in

human breast cancer. However, this will not reduce the values of each animal model to study specific aspect of the human breast cancer.

Although animal models for the breast cancer are available in various mammalian species, such as monkeys (Zhou, *et al.*, 2000), dogs and cats (Vail and MacEwen, 2000), the majority of experimental models are limited to the rodent species. It is because many rodent strains could develop spontaneous mammary tumors, and respond to a variety of carcinogenic agents such as hormones, chemicals, radiations, viral or transgenic factors, with development of either hormone-dependent or -independent mammary tumors, all of which show striking resemblance on the histopathology to the human breast cancer. The most common rodent models for the breast cancer include spontaneously developed, carcinogen-induced, virus-induced, hormone-induced, radiation-induced rodent models as well as the transgenic mouse model and the human tumor xenografts, with each model possessing its own advantages and limitations.

1.5.1 Mouse Models

The mouse models for human breast cancer include a variety of transgenic mice, gene deletion mice and naturally mutated mice. The analysis of transgenic mice, which express regulatory genes in a deregulated fashion, has provided insight into the function of these genes in the development of normal breast and breast cancer. On the other hand, the establishment of gene deletion or knockout model has identified genes which play critical roles in mammary gland development and carcinogenesis. Finally, the natural mouse mutants that exhibit altered mammary gland development have also provided useful information on the oncogene basis of the breast cancer.

In contrary to the rats, there are number of mouse strains which are susceptible to virus infections and develop mammary tumors in these mice. The mouse mammary tumor virus (MMTV) is the best example of these virus-induced mice models. MMTV is able to infect the neonatal female mice through the foster rat's milk. The infected female mice will develop preneoplastic hyperplastic alveolar nodules as early as in 4 weeks of age. The virus induced murine mammary tumors usually exhibit strong hormone dependence on estrogen, progesterone (Osborne *et al.*, 1990) and prolactin (Welsch and Gribler, 1973).

Although the murine models have contributed much to the present understanding of insertional mutagenesis and oncogene activation in the mammary tumorigenesis (Callahan, 1996), there are only a few corresponding human homologues are found mutated in human breast cancer. It has been criticized that these murine models may not necessarily represent the exact genetic events occurring in human breast cancer.

1.5.2 Rat Models

In the present study, expression patterns of hormone receptors in three Noble rat models are investigated. They include the chemical-induced model, hormone-induced model and the spontaneously developed models. In the following sections, a detailed review on these three models will be given.

1.5.2.1 Carcinogen Induced Rat Models

The carcinogen-induced rat model represents the most popular experimental systems used for studying human breast cancer. In this model, mammary tumors are usually induced in the Sprague-Dawley (SD) rat by 7,12-Dimethylbenz[a]anthracene

(DMBA) or in the SD rat or Fischer 344 rat by *N*-Methyl-*N*-Nitrosourea (NMU). The usage of DMBA was first described by Huggin in 1961 (Huggins & Yang, 1962) whereas NMU has been used increasingly in the last two decades (Thompson & Meeker, 1983). In both models, a single dosage of chemical carcinogen is usually administrated to the animals, either by gavage as for the DMBA or by intravenous or intraperitoneal injection as for the NMU. The tumor latency period generally ranges between 8 to 21 weeks, and with final tumor incidences near to 100 % (Russo *et al.*, 1990). The advantages of this animal model, such as ease in tumor induction, short tumor latency period and high tumor incidence, make this rat model popular for the study of human breast cancer.

It was also reported that the susceptibility of the rat mammary gland to either the DMBA or NMU-induced carcinogenesis is strongly dependent on the age of rats. A maximal tumor incidence will only be obtained when the chemical is administered to the virgin animals at the age of sexual maturity, that is between the age of 7 and 8-weeks-old. The reason for this observation is still uncertain, but may be related to the active proliferation of mammary gland during this maturity period.

With unknown genetic reasons, different rat strains show a different and deviated susceptibility to DMBA- or NMU-induced mammary tumorigenesis. Among the most commonly used rat strains, SD and Wistar-Furth are the most susceptible; Fischer 344 and ACI rats show intermediate susceptibility, whereas Copenhagen rats essentially completely resistant to the carcinogen treatment (Russo *et al.*, 1999). It was reported that Noble rats are also susceptible to the chemical treatment, with a high induction yield of adenocarcinoma, occasionally

fibroadenoma but also associated with the incidence of leukima (Noble and Cutts, 1959; Carroll and Noble, 1987).

Although there is the close resemblance of the tumor behaviors induced by the carcinogens, DMBA and NMU, some differences between the properties of these two chemicals are noted. Unlike NMU, DMBA requires metabolic activation for its carcinogenicity (Singletary, 1990). Several tissues are capable of activating DMBA. However, the mammary and hepatic activation are believed to be the most important factor for the DMBA-induced tumorigenesis. In contrast, NMU does not require metabolic activation, and it is frequently associated with *ras* gene mutations (Bos, 1989). Mutations of *ras* gene seldom occur in the DMBA-induced breast tumors. These discrepancies between DMBA and NMU suggest that the cellular signaling in the mammary carcinogenesis induced by DMBA and NMU are different.

The carcinogen models possess several characteristics that closely mimic to its human counterparts. Previous studies show that rats having completed a full-term pregnancy and lactation before carcinogen treatments exhibit a reduced incidence of the induced tumor (Russo *et al.*, 1990). In human, epidemiological studies have indicated that full-term pregnancy and lactation is protective against the breast cancer risk (Martin and Weber, 2000). On the other hand, many of the rat mammary tumors arisen from carcinogen induction are well-differentiated adenocarcinoma, of which the histopathology is highly similar to a significant proportion of human breast tumors (Russo *et al.*, 1990). Furthermore, the chemically induced tumors are generally responsive to estrogen and prolactin (Mershon *et al.*, 1995), with subsequent development into hormone-independence. Although the role of prolactin

is still not evident in human breast cancer (Vonderhaar, 1999), the loss of prolactin responsiveness is also frequently reported in the rat mammary tumors.

Human breast is an endocrine-dependent organ. In daily life, it has little chance to come in contact with or only has low exposure levels to chemical carcinogens. Therefore, it is not surprising that in some aspects, the behaviors of the chemically induced rat mammary tumors are different from their human counterparts. For instance, in the carcinogen-induced rat mammary tumors, there is a high incidence of mutations on certain oncogenes, such as the *ras* activation in the NMU-induced carcinogensis, and they are very rare in human breast cancers (Bos, 1989; Zhang *et al.*, 1990).

1.5.2.2 Hormone Induced Rat Models

As stated above, the physiological development of normal breast and the malignant progression of mammary cancer are tightly regulated by hormones, particularly the estrogen. This provides the basis for the hormone-induced rat model. As compared with the chemical induced model, in which the malignant transformation of mammary gland is initiated by chemical carcinogen, hormone-induced rat models are more closely mimic to the pathological conditions of the human breast carcinogenesis, as only naturally occurring hormones are used to induce the incidence of mammary cancers.

In many female rat strains, continuous exposure to estrogen alone can lead to the development of mammary tumors (Noble and Cutts, 1959). ACI rat, which is an inbred line derived from a cross between the August and Copenhagen strains, appears unique among all the rat strains as it is highly susceptible to estrogen-induced mammary tumorigenesis but rarely develops mammary cancers

spontaneously or in response to the chemical carcinogen or ionizing radiation (Harvell *et al.*, 2000). The ability of female ACI rats to develop high incidence of mammary carcinoma in response to the synthetic estrogen diethylstilbestrol (DES) was first noted by Dr. Dunning WF as early as in 1947 (Dunning *et al.*, 1949; Noble and Cutts, 1959). Recently, it was reported that ACI rats are also sensitive to the naturally occurring estrogen, 17β-estradiol, and results in a high mammary carcinoma incidence within relatively short time (Shull *et al.*, 1997). Interestingly, with uncertain reasons, its genetically closely related strain, Copenhagen rat (COP) is found to be resistant to the mammary tumor induction, no matter by the estrogen, chemical carcinogen or the ionizing radiation (Korkola and Archer, 1999).

More than four decades ago, Dr. Noble RL and his colleagues had conducted a series of detailed and influential studies on the induction of malignant mammary tumors in both male and female Noble rats employing subcutaneously implanted estrone pellets as the only inductive agent (Noble and Cutts 1959; Cutts, 1964; Cutts and Noble, 1964; Noble *et al.*, 1975). According to their pioneering studies, Noble rats was chosen because this rat strain showed a consistently high susceptibility to the estrone treament and is well tolerated to the hormone pellets. Although SD rats also showed estrone susceptibility, they tolerated the treatment poorly and mortality was high. On the other hand, Fischer and Wistar rats were found to be tolerated to the hormone well but they only showed low mammary tumor incidence. Similarly, Lewis rat strain showed comparable tumor incidence as that of the Fischer and Wistar rats but was intolerant of the drug and frequent death was resulted.

In his pioneering study, Dr. Noble reported that the natural incidence of mammary tumors in Noble rat strains was very low. However, after estrogenization,

the incidence of mammary tumors became more common in both sexes, particularly in the aged animals. The average latency period for the estrogen-induced tumor was 9 to 11 months, with incidence ranged from 60 to 80 %. However, increasing the estrone dosage was unable to alter both the tumor latency period and the incidence rate. Although benign fibroadenomas were occasionally collected, the majority of the induced mammary tumors were of epithelial origin, being various types of malignant carcinoma. Unlike the chemically induced mammary tumors, estrogen-induced carinomas sometimes showed metastasis to other secondary tissue sites including lymph nodes, liver and lung. The histopathology of the tumor cells is highly heterogenous, in which different histological types are often noted within the identical microscopic view of the same tumor. It was also reported that the induced tumors are hormone-dependent, removal of the estrogen markedly inhibits the growth of the neoplastic cells and is followed by tumor regression, whereas reinsertion of estone pellets usually resulted in tumor reappearance. Although multiple tumors were frequently found in individual Noble rats, the estrogen-induced mammary tumors usually appeared singly in each mammary gland (Noble and Cutts 1959; Cutts, 1964; Cutts and Noble, 1964; Noble *et al.*, 1975).

Although there are studies demonstrating that androgen inhibits normal breast development and suppresses the growth of natural occurring (Labrie *et al.*, 1992), carcinogen-induced (Costlow *et al.*, 1976, Dauvois *et al.*, 1989) and estrogen-induced mammary tumors (Dauvois *et al.*, 1991; Zhou *et al.*, 2000), simultaneous treatment with both androgen and estrogen in both male and female Noble rats was found to be able to induce high incidence (over 50 %) of mammary tumor within relatively short time (6 to 9 months) (Liao *et al.*, 1998; Xie B *et al.*, 1999). The Noble rat cancer model has demonstrated that androgen can pose a promoting effect

in the mammary carcinogensis, as the addition of androgen treatment was found to be able to shorten the tumor latency period significantly. These recent findings have greatly improved the values of Noble rat as an animal model for the study of human breast cancer. In addition, Noble rat can provide the researchers not only a model for the study of male breast cancer but also an appropriate experimental system to study the ambiguous role of androgen in mammary carcinogenesis.

1.5.2.3 Spontaneously Developed Rat Models

Spontaneous mammary tumors are not uncommon in rats, particularly in the aged female populations. The most commonly used rat strains, which are allowed to live their normal life span, show a significant high incidence of spontaneous mammary tumors (Table 1.3).

According to the pioneering studies by Dr. Noble RL, spontaneous mammary tumors were rare in Noble rats, although the tumors developed more commonly in animals over 1 year of age. Dr. Noble reported that in a Noble rat colony of 565 females and 299 males, the incidence of benign fibroadenoma in female Noble rats below 1-year old was about 8 % while that of the carcinoma was 1 %. In those females aged over 1 year, the incidence of the fibroadenoma sharply increased to 22 % whereas the carcinoma incidence risen to 3 %. In contrast, the incidence of spontaneous mammary tumors in male Noble rat was not evident, only 2 % of the animal was found bearing the mammary fibroadenoma (Noble *et al.*, 1975). These figures demonstrate clearly that age and sex are the two most important factors influencing the incidence of spontaneous mammary tumors. Although exact data may vary from one rat strain to others, spontaneous tumors develop more commonly in the aged animals, normally when the rat is over 1-year-old. The figures also

indicate that female animals are much more susceptible than males to develop spontaneous mammary tumors (Table 1.3). The reasons for this observation are unclear. It may be attributable to the lifetime accumulations of carcinogenic mutations in the aged animals and the high systemic estrogen levels in the female animals. Unlike the murine model, no virus is found to be able to induce mammary tumors in rat species so far (Noble and Cutts, 1959).

The Table 1.3 summarizes the incidence of spontaneously developed mammary tumors as reported by different laboratories. However, there are variations of the tumor incidences reported between different and even in the same laboratories. For instance, in the female SD rats, spontaneous mammary tumor incidences of 55 %, 62 %, 64 % and as high as 85 % have been reported. The tumor incidences in male SD rats also range from 19 % to 44 % (Sher, 1972). It is believed that lot of factors affect the spontaneous tumor incidence. These includes habitat environment such as the dietary content, the bedding, the temporal length of dark/light cycle, background radiation and other factors such as the presence of carcinogen or virus, the length of study, the age and mortality of the animals, the sample numbers, the germline mutations of the rat colony (Noble and Cutts, 1959; Sher, 1972).

Although there are variations in the incidences reported on the spontaneously developed mammary tumors, some rat strains are resistant to the induction of breast tumors and they only show a low incidence rate of the spontaneous mammary tumors. Generally, breast tumors accounts for 30 to 95 % of all the spontaneous tumors found in the female animals, as reported in the female SD, Wistar and August rats. However, in Copenhagen rats, which are resistance to mammary tumorigenesis,

only a 5.6 % of the spontaneously developed tumors are of mammary origins (Noble and Cutts, 1959).

As compared to all the models induced by carcinogens or hormones, the whole process of carcinogenesis in the spontaneously developed model is naturally occurring and without any exogenous factors. This model is closely mimic to the mammary carcinogenesis in human. Apart from its close resemblance to human breast cancer, the spontaneous developed model is also an excellent experimental system to study the early neoplastic transformation of the mammary gland. In contrast to the chemical or hormone-induced models, in which malignant tumors are common, the spontaneous developed tumors collected in our colony frequently show hyperplastic and dysplastic samples as well as a high proportion of begin adenoma or fibroadenoma, although occasionally highly malignant carcinomas were also collected. It is believed that the spontaneously developed model could provide a useful model to elucidate a complete picture on the malignant transformation events occurring during the mammary gland carcinogenesis from the early hyperplasia to the invasive carcinoma. This greatly facilitates our understanding on the human breast cancer.

In spite of the above-mentioned advantages, the incidence rates of the spontaneous mammary tumors are low and uncertain. Large numbers of animal should be maintained to their normal life span in order to collect significant quantity of the spontaneous mammary tumors for analysis and the costs involved are prohibitively expensive to most laboratories. Due to this reason, the experimental use of the spontaneously developed models is still limited.

1.6 Aims of Study

Noble rat is a valuable animal model for the study of human breast cancer in addition to prostate cancer. On one hand, this rat strain is susceptible to the hormonal treatment. On the other hand, according to our unpublished experience, Noble rat is also sensitive to the carcinogen induction and is able to develop mammary tumors spontaneously in a high incidence. Therefore, Noble rat strain can offer the researchers a valuable tool to study the similarity and difference between the phenotypic behaviors of the mammary tumors induced by various treatments. In the present study, the incidence rates, lengths of latency period and histopathology of the spontaneously developed, hormone-induced and carcinogen-induced mammary tumors of female Noble rats were compared. In order to investigate the involvement of different hormones in the mammary gland carcinogenesis, the expression patterns of estrogen receptor (ERα & ERβ), progesterone receptor (PR), androgen receptor (AR) and prolactin receptor (PRLR) in these spontaneously developed, hormone-induced and carcinogen-induced breast tumors were also characterized and compared by immunohistochemistry and western blotting. The comparison on the phenotypic behaviors of the spontaneously developed, hormone-induced and carcinogen-induced mammary tumors are believed being able to provide us insights on the underlying carcinogenic mechanisms on the mammary glands in these three different rat models.

Table 1.1 Worldwide Incidences of Cancers in Women in 1980
(McPherson *et al.*, 2000)

Anatomical Sites of Cancer	Number of Cases (per 1000s)	Percentage of Total
Breast	572	18
Cervix	466	15
Colon and Rectum	286	9
Stomach	261	8
Endometrium	149	5
Lung	147	5
Ovary	138	4
Mouth and pharynx	121	4
Oesophagus	108	4
Lymphoma	98	3

Table 1.2 Risk Factors for Breast Cancer
(McPherson *et al.*, 2000)

Risk Factor	Relative Risk	High Risk Group
GENETIC		
Family History	≥2	Breast cancer in first degree relative when young
HORMONAL		
Age at menarche	3	Menarche before age 11
Age at menopause	2	Menopause after age 54
Age at first full pregnancy	3	First child in early 40s
Taking exogenous hormones:		
Oral contraceptives	1.24	Current use
Hormone replacement therapy	1.35	Use for ≥10 years
Diethylstilbestrol	2	Use during pregnancy
Obesity:		
Premenopausal	0.7	Body mass index >35
Postmenopausal	2	Body mass index >35
OTHERS		
Age	>10	Elderly
Geographical location	5	Developed country
Diet	1.5	High intake of saturated fat
Radiation exposure	3	Abnormal exposure in young females after age 10
Alcohol consumption	1.3	Excessive intake

Table 1.3 Incidence Rates of Spontaneous Mammary Tumors in Common Rat Strains

Rat Strains	Spontaneous mammary tumors	Incidence Rate	
		Female	Male
Sprague-Dawley (Chandra *et al.*, 1992)	Fibroma	0.3%	-
	Fibroademoma	18.96%	0.75%
	Adenoma	3.54%	0.60%
	Adenocarcinoma	8.8%	0.15%
	Total Incidence	31.3%	2.3%
Wistar (Walsh and Poteracki, 1994)	Fibroademoma	25.26%	1.13%
	Adenoma	4.38%	0.15%
	Adenocarcinoma	13.14%	1.02%
	Total Incidence	42.78%	2.3%
Fischer 344 (Chandra and Frith, 1992)	Fibroademoma	11.1%	-
	Adenoma	1.9%	-
	Adenocarcinoma	1.6%	-
	Total Incidence	14.6%	0%
Noble (Noble *et al.*, 1975)	Fibroadenoma	22%	2%
	Carcinoma	3%	0%
	Total Incidence	25%	2%

Figure 1.1 International variation in age-adjusted female breast cancer from 1988 to 1993 (Parkin *et al.*, 1997)

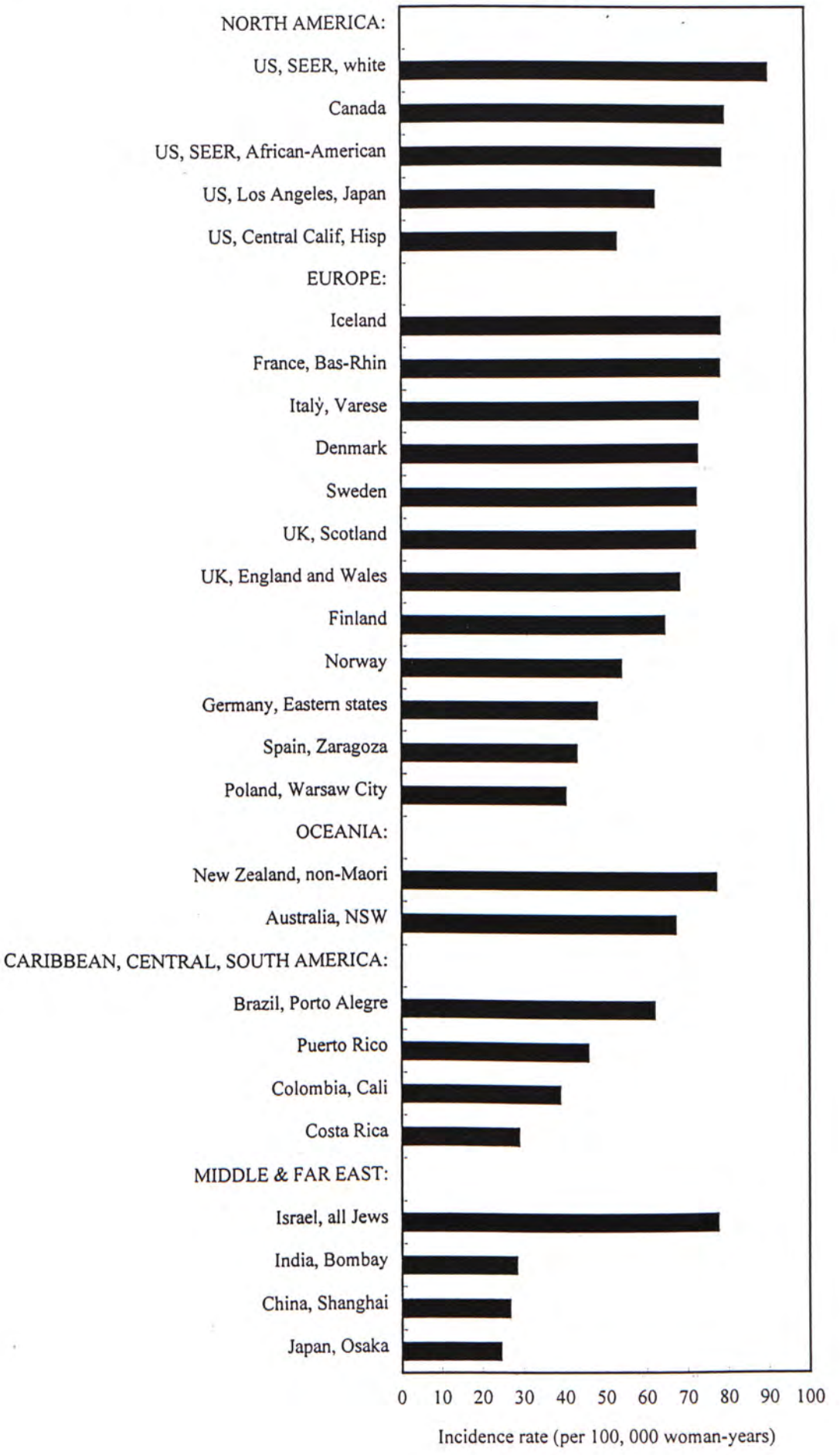


Figure 1.2 Incidence Rate of Five Most Common Female Cancers in Hong Kong
(Source: Hong Kong Cancer Registry, 1996)

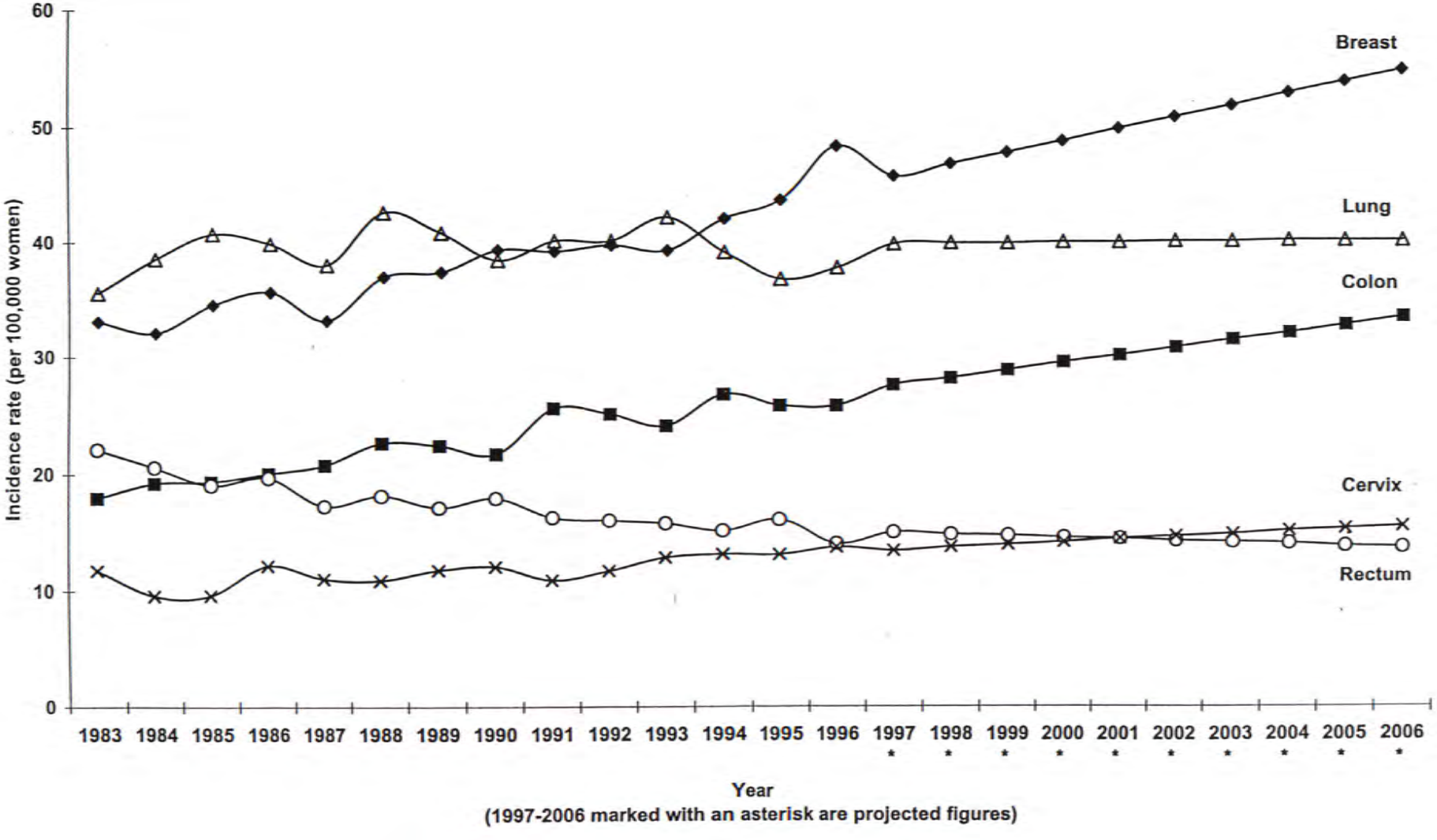


Figure 1.3 Incidence and Death Rate of Female Breast Cancer in Hong Kong
(Source: Hong Kong Cancer Registry)

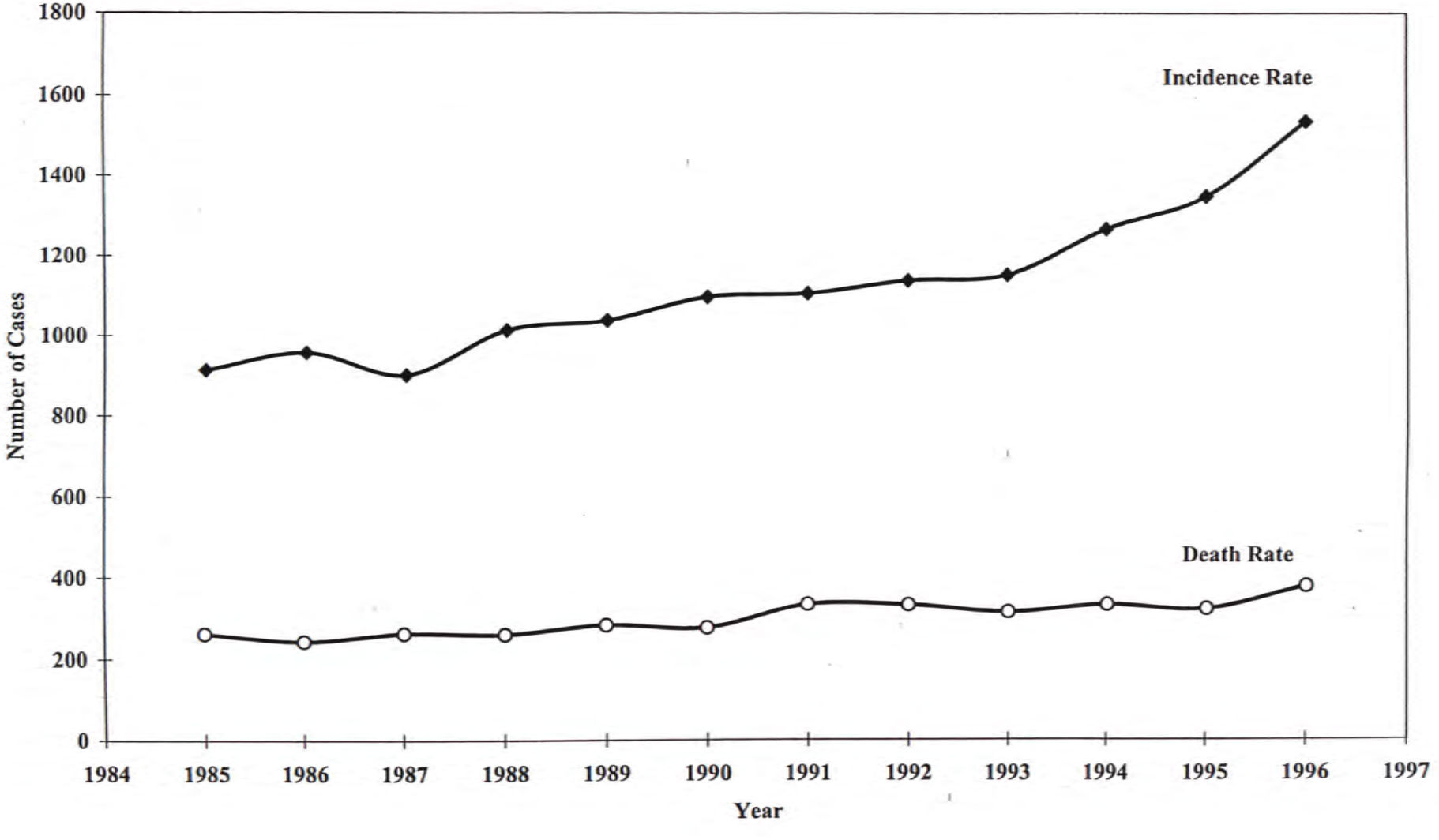


Figure 1.4 Molecular Mechanisms of Steroid Hormones

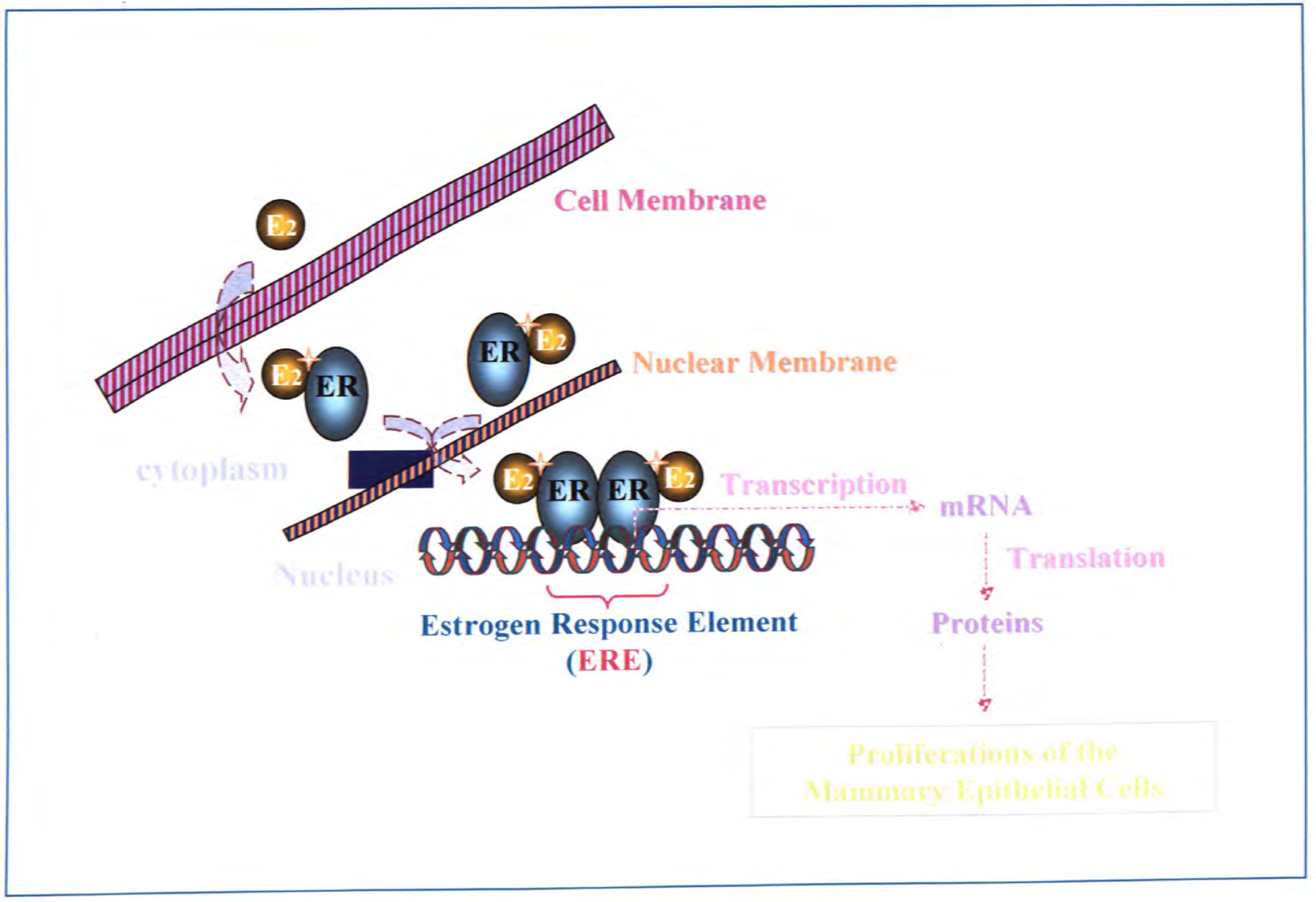
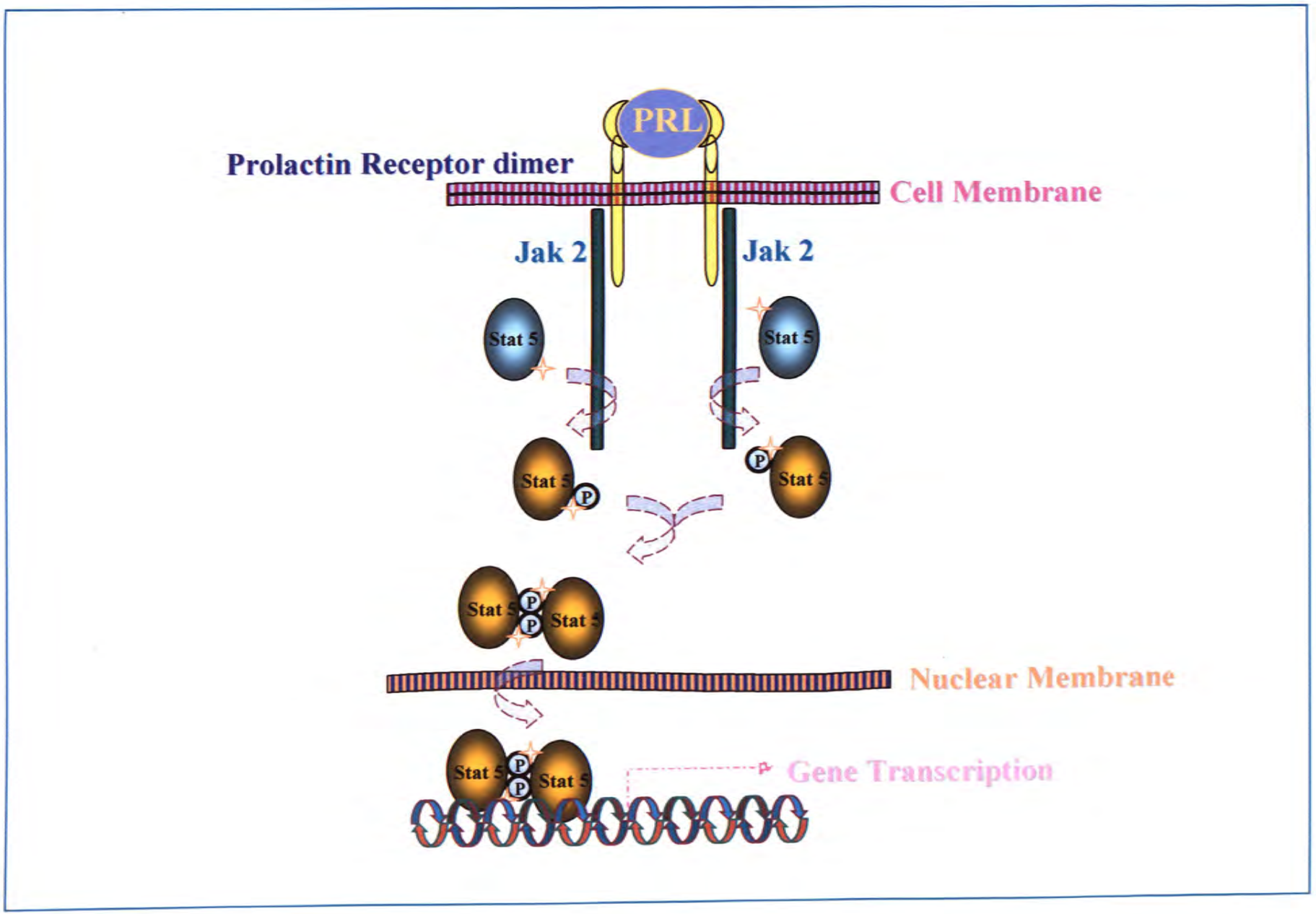


Figure 1.5 Molecular Mechanisms of Prolactin



Chapter 2. Materials and Methods

2.1 Origin and Supply of Noble Rats

The black-hooded Noble rat strain was originated from Dr. JB Collip's laboratory at McGill University in 1940s. This rat colony was believed to arise from outcross between the Long-Evans rats and the Wistar rats, and the Long-Evans rats were from Dr. Philip Smith at Columbia University. Subsequently, Dr. RL Noble of the Cancer Research Centre in Vancouver further developed a distinct inbred Noble rat strain from this original stock. The rat strain which Dr. RL Noble successfully developed was of random littermate breeding and was characterized by black-hooded, moderately sized, readily breeding and extremely docile, thereby ideal for experimental works. In 1970s, the Medical Research Council (Carshalton, England) assigned this colony as Noble (Nb) rat after evaluation on its genetic composition and degree of inbreeding contributed by Dr. RL Noble. The offspring was then randomly bred for later experiments (Noble *et al.*, 1975).

In the present study, breeders of Nb rats were obtained originally from the Charles River Breeding Laboratories in Japan in 1996. The strain is then being maintained by the Laboratory Animal Service Centre, the Chinese University of Hong Kong. Young adult animals of suitable age were used in the present study. In addition, female Sprague-Dawley rats were also used for the induction of mammary tumors by *N*-methyl-*N*-nitrosourea (NMU).

2.2 Supply of Materials

Unless specifically stated, all the chemicals used in the present study were obtained from Amersham Pharmacia Biotech. (NJ, USA), Boehringer Mannheim

(Mannheim, Germany), Fluka (Buchs, Switzerland), GibroBRL (USA) and Sigma Chemical Company (St. Louis, USA).

The antibodies used were obtained from Affinity Bioreagents (Colorado, USA), Jackson ImmunoResearch Labs (USA), NeoMarkers (California, USA), Santa Cruz Biotechnology (California, USA) and Zymed Labs (San Francisco, USA).

2.3 Induction of Mammary Tumors by Singe Dose of Chemical Carcinogens in Female Rats

2.3.1 Induction by 7,12-Dimethylbenz[a]anthracene in Female Noble Rats

Female virgin Noble rats at 7 to 8 weeks of age were used. The carcinogen 7,12-Dimethylbenz[a]anthracene (DMBA; Sigma, USA) was dissolved in corn oil at a concentration of 10 mg/ml. Single dose of DMBA emulsion was administrated into the Noble rat intragastrically at a dosage of 50 mg/kg body weight using a 16G curved blunt-ended gavage needle. The control rats were administered with corn oil only. The treated rats were then housed in cages and fed on normal chows and drinking water *ad libitum*. After treatment for 2 months, the animals were palpated regularly for the detection of mammary tumors. The animals were killed when they became morbid or when obviously palpable lumps of mammary tumors were detected in the mammary gland. The mammary tissues were then collected for subsequent studies.

2.3.2 Induction by *N*-Methyl-*N*-Nitrosourea in Female Sprague-Dawley rats

Female Sprague-Dawley rats of 7 to 8-week old were obtained from the Laboratory Animal Service Centre, the Chinese University of Hong Kong. Upon arrival, the carcinogen *N*-Methyl-*N*-Nitrosourea (NMU; Sigma, USA) was stored at 4°C in dark. A 20 mg/ml NMU solution was freshly prepared by dissolving the chemical in normal saline (0.85% NaCl) containing 0.05% acetic acid. Only freshly

prepared NMU solution, which was used within 30 minutes after preparation, was used for the tumor induction. A single dose of NMU solution was then injected intraperitoneally at the lower left quadrant of the rat abdomen at a dosage of 50 mg/kg body weight. Age matched untreated rats were used as controls for the experiment. The rats were then fed on normal chows and drinking water *ad libitum*. After NMU administration for over 2 months, the animals were palpated regularly for the detection of mammary tumors. Suspected mammary lumps were then excised from the animals for further analysis.

2.4 Induction of Mammary Tumors by Long-Term Treatments with Steroid Hormone

2.4.1 Preparation of Steroid Hormone-filled Silastic®Tubings

Silastic®laboratory tubings of inner diameter of 1.67 mm and outer diameter of 3.18 mm (Catalogue Number 508-008; Dow Cowing; Michigan, USA) were cut into lengths of either 1 cm or 2 cm. The 2-cm tubings were tightly filled with powder of testosterone (T; Sigma, USA) while the 1-cm tubings were packed with either 17β-estradiol (E_2; Fluka, USA) or diethylstilbestrol (DES; Sigma, USA) using an air pump. Both ends of the steroid hormone-filled tubings were then sealed with the Silastic®medical adhesive (Silicone type A; Dow Corning). When the sealing adhesive became hardened, tubings were soaked overnight in 70% ethanol in order to ensure complete sealing of tubings and removal of hormone powder residues adhered to the tubing surface. The tubings were then immersed in 0.5M phosphate buffered saline (PBS) for another 2 days. Finally, the tubings were blotted dry with Kimwipes tissue, stored at 4°C and were ready for surgical implantation into the Noble rats.

3.4.2 Surgical Implantation of Silastic®Tubings

Virgin female Noble rats of 7 to 8 weeks of age were used. The animals were anaesthetized by intraperitoneal injection of 7 % chloral hydrate solution at a dosage of 0.5 ml/100g body weight. The implantation site was located over the skin between the scapulae and the skin was shaven with an electric clipper. The hairless operating area was then cleaned with 70 % alcohol. The skin was cut open and a subcutaneous pocket was made by blunt dissection beneath the skin in a caudal direction. Blunt forceps were used to make the tubing accommodation, in which testosterone-implants (T) were inserted to the left scapula whereas the estrogen-implants (DES or E_2) were placed on the right side. After implantation, the tubings were pushed subcutaneously into the inguinal region. The incision was sutured and sprayed with a permeable wound dressing (Smith & Nephew Medical Limited, England). The animals were housed in cages and fed on normal chows and drinking water. After the tubing implantation, regular palpation of the animals was made for mammary tumor detection.

2.4.3 Protocols of Hormonal Treatments

The animals under hormonal treatments were divided into two groups, namely combined T+E_2 group and combined T+DES group. For combined T+E_2 group, two 2-cm testosterone tubings and one 1-cm 17β-estradiol tubing were implanted subcutaneously (Leav *et al.*, 1988). For combined T+DES group, similar protocol was applied but diethylstilbestrol tubings instead of 17β-estradiol tubings were implanted into the rats. For the control group, age-matched intact virgin female Noble rats, without tubing implantation were used.

2.5 Collection of Spontaneously Developed Mammary Tumors in Noble Rats

About 3-mouths-old intact adult male or virgin female Noble rats were housed in cages with room lighting on 12 hours light/dark cycle. The animals were fed on normal chows and drinking water *ad libitum*. Routine observation was made to check for morbidity and mortality. The animals were palpated regularly up to 2 years and were killed when they became morbid or when obviously palpable lumps were detected in the mammary gland.

2.6 Transplantation of the Spontaneously Developed Mammary Tumors into Noble Rats

Intact aged female or male Noble rats bearing spontaneously developed mammary tumors were regarded as donor animals for the tumor transplantation. On the other hand, intact Noble rats of the same sex as the donor animals were used as host animals receiving the spontaneously developed mammary tumors. The host animal was adult rat of about 3 months old. Both the tumor-bearing donors and the host Noble rats were anaesthetized by intraperitoneal injection of 7 % chloral hydrate solution. The tumor transplantation site was made over the skin between the scapulae of the host Noble rats. The operating area was shaved with an electric clipper and then cleaned with 70 % alcohol. An incision was made and a small subcutaneous pocket was created by using a pair of blunt-ended scissors under the skin in a caudal direction. On the other side, the spontaneously developed mammary tumor was then quickly dissected from the donating Noble rat and rinsed briefly with saline in a Petri dish. It was then immediately transferred to a second dish containing Hank's saline. All extraneous tissue adhered to the tumor was removed and only the solid viable tumor tissue was selected for transplantation. The selected tissue was transferred

quickly to a third Petri dish containing Hank's buffer, in where the tumor was cut into small pieces with size of about 5 mm^2 in size for subsequent transplantation. A fat pad was then extracted from the subcutaneous pocket of the host animals. A minute cavity was made in the fat pad. One to two pieces of tumor fragment were transferred to the fat pad cavity with a pair of fine forceps. Subsequently, the tumor-containing fat pad was carefully pushed back into the subcutaneous pocket, in a position as far from the skin incision site as possible in order to prevent any loss of the tumor pieces. After transplantation, the incision sites on both the donator and host animals were sutured and sprayed with a permeable wound dressing. The animals were housed in cages and fed on normal chows and drinking water *ad libitum*. The rats were inspected routinely in order to check the conditions of the donating animals and the growth of the transplanted tumor pieces in the host rats

2.7 Bilateral Ovariectomy of Female Noble Rats Bearing Spontaneously Developed Mammary Tumors

Female Noble rats bearing spontaneously developed mammary tumors were ovariectomized bilaterally. The animal was first anesthetized by intraperitoneal injection of 7 % chloral hydrate solution. The anaesthetized animal was then laid ventrally. A small incision of about 1-2 cm was made on the midline dorsal skin, at a position of about halfway between the hump of the back and the base of the tail. Scissors were then inserted subcutaneously through the skin incision. Blunt dissection was carried out on skin beneath on either side of the incision for a short distance, in order to access to the abdominal muscles under the skin. The skin incision was retracted with forceps, first to one side and the abdominal muscle wall was cut open in order to get into the abdominal cavity. The ovary was exposed and ligated before excision. Similar procedure was done on the other side and the other

ovary was excised too. Approach into the abdominal cavity on the either side was made by making a small cut of the abdominal muscle wall. The muscle incisions were located half to two-thirds of the way down the rat's spinal column. The ovary, which was embedded in a variable amount of fats, could be exposed easily from the muscle incision. After successfully exposing the ovary, its periovarian fat was gently grasped. The ovary was then carefully pulled out from the animal's abdominal cavity. The junction between the Fallopian tubes and the uterine horn was ligated. The ovary, with all the accompanying blood vessels and periovarian fat, were excised over the fallopian tubes beyond the ligation site. The uterine horn was then returned into the animal's abdominal cavity. Subsequently, the muscle incision was sutured. The skin incision was then retracted with forceps to another side to remove the remaining ovary. Identical procedures for removal of the ovary were followed. After bilateral ovariectomy, the skin incision was sutured and sprayed with a permeable wound dressing. The animals were housed in cages and were fed on normal chows and drinking water. Routine inspection was made in order to check the post-surgery recovery of the animals and the growth of the transplanted spontaneously developed mammary tumors in the animals.

2.8 Measurement of Mammary Tumor Growth

After ovariectomy, the size of the spontaneously developed mammary tumors in the animals was determined weekly. Each dimension of the tumor diameter was measured three times using an electronic digital caliper. Result was expressed as tumor volume, which could be obtained using the following formula (Boven *et al.*, 1985):

$$\text{Mammary Tumor Volume} = \tfrac{1}{2} \times \text{length} \times \text{width} \times \text{height}$$

2.9 Whole Mount Preparation of the Hormone-Treated Mammary Glands in Noble Rats

This whole-mount protocol was provided by Dr J Russo, Breast Cancer Research Laboratory, Fox Chase Cancer Center, Philadephilia, USA. The hormone-treated Noble rats were anesthetized with an intraperitoneal injection of 7% choral hydrate solution. The animals were placed on its back with the four extremities stretched on a surgical board. The skin was opened by a longitudinal midline incision extending from the submaxillar to the public regions. The skin was separated from the muscle by gently pulling the skin and blunt dissection with scissors. Fixation for whole mount preparation was obtained by removing the skin pelt with all the mammary glands attached. The skin pelt was stretched and pinned to a corkboard and then immersed in 10% neutral buffered formalin for 24 hours. After fixation, the mammary glands were identified by locating the corresponding nipple. They were then dissected starting the nipple and processed dorolaterally. The mammary fat pad was lifted with forceps. It was separated from the subcutaneous tissue by dissection with iris scissors or a scalpel. Tissues were defatted by submersion in acetone for 2 or more days. The acetone was changed when it became cloudy. The containers with glands and acetone were placed on a shaker bath. All the defattening procedure was preformed under continuous agitation at approximately 80 rpm. The tissues were hydrated in decreasing concentrations of ethanol: 100 %, 95 % and 70 % for 1 hour, each with continuous agitation before processing with the staining in toluidine blue.

The whole process of staining and washing of the mammary tissues were carried out in continuous agitation. The tissues were stained in 0.025% Toluidine Blue solution for 2 hours. The tissues were washed in distilled water for 30 minutes. The tissues were then destained in pure methanol and 70 % ethanol for 30 minute respectively. The tissues were subsequently washed in distilled water and fixed in 4

% ammonium molybdate for 30 minutes. The tissues were washed again in distilled water for overnight. The tissues were then dehydrated in 70 %, 95 % and 100 % ethanol for 1 hour each. The tissues were transferred to xylene overnight and finally mounted to slides.

2.10 Histological Examination of the Mammary Gland and Tumors in Noble Rats

The Noble rats of different treatment groups were palpated regularly for the inspection of mammary tumors. The animals were killed when obviously palpable lumps were detected in the mammary gland. The palpable mammary lumps were then quickly excised from the anaesthetized animals. Abdominal mammary glands were also collected form the normal and lactating untreated Noble rats as a control comparison. The excised tissues were fixed by immersion overnight in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. The fixed tissues were washed thoroughly in running water, dehydrated through graded ethanols, cleared in xylene and embedded in paraffin. Paraffin sections of 5μm in thickness were cut and stained with hematoxylin and eosin (H&E). After dehydration in alcohol and xylene, the sections were mounted in Permount® for light microscopic study. The mammary tumors were graded histopathologically using the criteria as outlined by Dr. Jose Russo and Irma H. Russo, Fox Chase Cancer Centre, USA (Russo & Russo, 1978, 1987; Russo *et al.*, 1977, 1979, 1982, 1983).

2.11 Detection of Protein Expression of Hormone Receptors in Normal Mammary Glands and Mammary Tumors of Noble Rats

2.11.1 Antibodies

The primary antibodies used for the immunohistochemical and Western blot analysis were as follows: a rabbit polyclonal androgen receptor (AR) antibody C19 raised against the last 20 amino acids of rat AR C-terminal (Santa Cruz Biotechology, California, USA); a rabbit polyclonal estrogen receptor α (ERα) antibody MC20 raised against the last 20 amino-acids of rat ERα C-terminals (Santa Cruz Biotechology, California, USA); a rabbit polyclonal estrogen receptor β (ERβ) antibody PA1-310B raised against to the C-terminal amino acid residues 467-485 of rat ER beta (Affinity Bioreagaents, Colorado, USA); a rabbit polyclonal progesterone receptor (PR) antibody C20 raised against the amino acids 545-564 of human PR, the segment differing from the same region of rat PR in two amino acids (Santa Cruz Biotechology, California, USA); a mouse monoclonal prolactin receptor (PRLR) antibody clone U5 specific to the extracellular portion of the rat liver PRLR (Affinity Bioreagaents, Colorado, USA); a mouse monoclonal prolactin receptor (PRLR) antibody clone T6 specific to the ligand binding site of the rat liver PRLR (Affinity Bioreagaents, Colorado, USA). A mouse monoclonal multi-keratin antibody clone C-11 (NeoMarker Carlifornia, USA), which could recognize the rat keratins 4, 5, 6, 8, 10, 13 and 18, were also used for immunohistochemical study in order to correlate the hormone receptor expression patterns with the mammary tumor histopathology.

For the immunohistochemistry, avidin-biotin peroxidase complex method (ABC method) was used to amplify the immunohistochemical signals on sections. The biotinylated secondary antibody used in the present study included goat anti-rabbit IgG, donkey anti-mouse IgG and donkey anti-goat IgG (Jackson Immunoresearch Labs, USA).

For Western blotting, an alkaline phosphatase-mediated colorimetric method was used to detect the antibody binding sites on the PVDF membrane. The alkaline phosphatase conjugated secondary antibodies used in the Western blot analysis included goat anti-rabbit IgG, goat anti-mouse IgG and rabbit anti-goat IgG (Zymed Labs, USA).

2.11.2 Immunohistochemistry

Normal Noble rat mammary glands or tumor tissues were fixed overnight in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. Other organs included lactating mammary gland, uterus, liver and prostate were also collected from normal Noble rats for fixation and were used as control tissues for immunohistochemistry. The fixed tissues were then embedded in paraffin. Paraffin sections of 5 μm in thickness were dewaxed in xylene and hydrated in graded ethanols. The hydrated sections were treated with 0.5 % hydrogen peroxidase in absolute methanol for 30 minutes in order to remove the endogenous peroxidase activity. The sections were then rinsed in phosphate buffered saline (PBS), pH 7.4, for 5 minutes. Antigen retrieval was preformed by heating the sections in 0.1M Tris-HCl containing 0.5 % urea, pH 9.5 for 10 minutes at 95°C. The sections were then remained in the same buffer solution for additional 15 minutes at room temperature and washed in PBS for 2 times, 10 minutes each. The sections were incubated in a blocking solution containing 0.1% bovine serum albumin (BSA) and 0.05% Triton X-100 in PBS for 30 minutes. The sections were incubated with the diluted primary antibody (1:200 in the blocking solution) in a moist chamber overnight at 4°C. For control sections, the sections were incubated in the blocking solution without the antibodies. After overnight incubation, the sections were washed in PBS with 3 changes, 15 minutes each. After washings, the sections were incubated with the biotinylated secondary

antibody for 1 hour at room temperature in a humid chamber. After the incubation, sections were rinsed with PBS for 15 minutes. The immunohistochemical signals on the sections were detected by the avidin-biotin peroxidase complex (ABC) method (Hsu and Raine, 1981). In brief, an ABC complex solution was prepared by mixing 10 μg avidin (EY Laboratories, San Mateo, USA) and 2.5 μg biotin-conjugated horseradish peroxidase (Jackson ImmunoResearch Labs, USA) in 0.05 M PBS. The ABC mixture was allowed to stay at room temperature for 30 minutes prior to applying to the sections. The sections were then incubated with the ABC complex for 1 hour at room temperature in a moist chamber. After incubation, the sections were washed in PBS for 3 changes, of 15 minutes each. The peroxidase activity was then visualized by a glucose oxidase-diaminobenzidine (DAB)-nickel intensifying procedure (Chan and Choi, 1995). In brief, the sections were first pre-incubated in an ammonium nickel sulfate-DAB solution (0.4 g ammonium nickel sulfate, 20 mg β-D-glucose, 4.0 mg ammonium chloride and 5.0 mg DAB in 10 ml 0.1 M acetate buffer, pH 6.0) for 10 minutes. Glucose oxidase (0.5 mg per 2.0 ml distilled water; Type VII, Sigma) was then added to the DAB solution. The positive reaction sites were demonstrated as a dark blue or black color. The reaction was stopped by incubating the sections in 0.1 M sodium acetate, pH 6.0, and subsequent washing in running tap water. Finally, sections were dehydrated in graded ethanols, cleared in xylene, mounted with Permount®and examined under the light microscope.

2.11.3 Protein extraction, SDS-PAGE and Western blotting analysis

Freshly dissected normal mammary glands or mammary tumors were immediately frozen in liquid nitrogen. The frozen samples were stored at –70°C until protein extraction. Multiple samples of equal amount of normal mammary gland or tumor tissues induced or developed by the same treatment were pooled

together from at least three individual animals. The pooled samples were homogenized (Polytron PT 2000, Brinkmann) in 10-fold volumes (per gram wet weight tissue) of cold lysis buffer in an ice-bath. The lysis buffer contained 20 mM PIPES, 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 10 mM monothioglycerol, 50 mM sodium fluoride, 0.5 mM sodium orthovanadate, 0.5 mM PMSF, 2 ug/ml aprotinin and 5 μM leupeptin. The homogenates were centrifuged at 12,000 r.p.m. for 20 minutes at 4°C. The supernatant was collected and its protein concentration was determined by the bicinchoninic acid protein assay (BCA protein assay, Pierce, USA) with bovine serum albumin (BSA) as standard. The quantified protein samples were aliquoted in small volume and kept at until electrophoresis (Liao *et al.*, 1998).

Before gel electrophoresis, the protein samples were first mixed with 0.2 volume of 2x sample buffer (0.125 mM Tris-HCl, pH 6.8, 5 % sodium dodecyl sulphate (SDS) [w/v], 20 % glycerol [w/v], 0.002 % bromophenol blue, 10 % 2-mercaptaethanol) and heated at 100°C for 5 minutes for denaturation. The denatured protein samples were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10 % gel using a mini-gel apparatus (Bio-Rad, USA). Proteins were loaded at 15 μg per lane. The gels were run at 100V constant voltage per slab for about 2 hours. Protein molecular weight markers were obtained from Bio-Rad Laboratories. For further confirmation of equal protein loading, gels were stained with Commassie blue. The gels were submerged in a 0.2 % Commassie blue staining solution (0.2 % Commassie blue in destaining solution) for about 10 minutes. The stained gels were washed in a destained solution (methanol: glacial acetic acid: distilled water, 3:1:6 v/v).

After electorphoresis, gels were fixed in a transfer buffer containing 25 mM Tris, 192 mM glycine and 10 % methanol, pH 8.3, for 30-45 minutes. Proteins were

electrophoretically transferred onto the PVDF membranes (0.20 μm, Schleicher & Schuell) in the transfer buffer at 100V at 4°C for 1 hour. The blotted membranes were blocked with 1 % Tween-20 in PBS for 1 hour and then washed in a washing buffer containing 0.05 % Tween-20 in PBS, for 30 minutes. The primary antibodies for hormone receptors were first diluted 1:1000 in PBS with 2.5 % non-fat milk powder. The membranes were subsequently incubated with the diluted antibody overnight at room temperature on a rocking platform. For control blots, membranes were incubated in 2.5 % non-fat milk in PBS without primary antibodies. After overnight incubation, membranes were washed with the washing buffer, followed by a one-hour incubation with the alkaline phosphatase-conjugated secondary antibody diluted 1:2000 in PBS with 2.5 % non-fat milk powder at the room temperature. After washing, the antibody binding sites on the blotted membranes were detected by a colorimetric method. The membranes were first incubated in a substrate buffer (100 mM Tris, 100 mM NaCl and 50 mM $MgCl_2$, pH 9.5) for 10 minutes. The color reaction was developed by incubation in a chromogen solution containing 45 μl nitroblue tetrazolium (NBT, 75 mg/ml in dimethylformamide), 35 μl 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, 50 mg/ml in dimethylformamide) and 0.24 mg/ml levamisole (1 mM) in 10 ml substrate buffer at 25°C for several minutes. The color reaction was stopped by incubating in a stop buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and then rinsed with running tap water. The color-developed membranes were finally dried with blotting paper.

Chapter 3. Results

3.1 Gross Appearance of Mammary Tumors

The rat mammary tissues extend from the auxiliary to the inguinal region and are distributed in pairs on either side of the ventral midline. There are totally six pairs of mammary glands. One pair of mammary gland can be found in the cervix, another two pairs in the thorax and abdomen respectively, and the remaining pair is located in the inguinal region (Figure 3.1.1A). Each mammary gland extends from the medially located nipples in the dorsolateral orientation (Figure 3.1.1B).

According to our results, mammary tumors could develop in Noble rats spontaneously or be induced by the hormonal or carcinogenic stimulations. The tumors could be found in any area of the mammary tissue. No particular tendencies for the localization of the mammary tumors on the animal were observed. In each tumor-bearing animal, more than one mammary tumor generally could be found. The neoplasm usually distributed singly in each mammary gland. However, in some cases, the tumors would arise in different positions of the same mammary gland.

Grossly, mammary tumors appeared as palatable lump in the mammary areas of the animals. Usually, they were firm, soft and smooth masses and were freely moveable in the subcutaneous tissue.

Mammary tumor could attain to huge size, occasionally could be as large as about half of the body weight of the animals. Gross dissection revealed that the large tumor often became necrotic and infected in the core region. The large tumor would also disrupt the ability of the animal to move around or even to eat. This further contributed to the debility and even mortality of the animals

Usually, but not always, there were some differences in the gross appearances of the mammary tumors under different treatments. Gross dissection

revealed that the incidence of spontaneously developed mammary tumors in Noble rats was usually associated with a plenty of milk secretion in the mammary tissues. The milk secretions usually appeared in the form of white patches in the mammary areas surrounding the tumors (Figure 3.1.2A). In some case, milk secretions could also be found inside the tumor. Apart from the mammary areas adjacent to the tumor, the milk patches also appeared in other mammary glands even tumors were not found in those areas. Generally the spontaneously developed mammary tumors are white in color, rubbery and firm in gross appearance. The neoplasic tissues usually shells out from the main mass when the tumor was being dissected.

On the other hand, the mammary tumors induced by the carcinogen DMBA and NMU rarely associated with the incidence of milk secretions (Figure 3.1.2B). The tumors generally appeared as solid masses and could be sectioned easily. In some cases, areas of necrosis and haemorrhage were encountered.

For the hormone induced mammary tumors, both the T+E_2 and T+DES induced group gave similar gross appearances. In comparison, the hormone induced mammary tumors were relatively small in size and difficult to discover. The hormone treated animals were usually moribund when the mammary tumors were palpable. In most cases, multiple prominent black lesions were found in the mammary areas nearby the tumors (Figure 3.1.2C). Occasionally, these lesions were located in glandular areas far away from the neoplasm. Gross dissections revealed that these black lesions were cystic structures containing blood and necrotic materials. The hormone-induced mammary tumors were also filled with fluids. In some case, the incidence of the hormone induced mammary tumors was associated with the abnormality of other organs. The female sex organs, uterus and ovary, were

frequently involved. Occasionally, the liver and lung of the animal were also affected.

3.2 Incidence Rate of Mammary Tumors

3.2.1 Spontaneously Developed Mammary Tumors in Noble Rats

Totally 40 female and 32 male Noble rats were remained intact and used as control animals for the experiment. In this control group, 27 mammary tumors were collected from 18 female (Table 3.1). One mammary tumor was also collected from the male rat. The incidence rate of the spontaneously developed mammary tumors in the female Noble rat was 45% while that of the male animals was 3.13%. The average age of the Noble rat for the incidence of spontaneous mammary neoplasm was 14-months-old. The incidence of the breast tumors was rare when the age of the animal was less than 12-months-old. Figure 3.2.1 showed a cumulative incidence of spontaneous mammary tumors in female Noble rats against the age of the animals.

3.2.2 Hormone Induced Mammary Tumors in Female Noble Rats

The incidence rate, latency period and distribution of the hormone induced mammary tumors in female Noble rats were given in table 3.2, 3.3 and 3.4.

For the testosterone and 17β-estradiol ($T+E_2$) treatment group, the mortality rate of the hormone treated Noble rats was 25% and the incidence rate of the hormone induced tumor was 46.66%. On the other hand, for the testosterone and Diethylstilbestrol (T+DES) group, the mortality rate was 55% and the incidence rate was 55.55%. In comparison, the mortality of T+DES group was more than twice the value of the $T+E_2$ group. The incidence rate of the T+DES induced mammary tumors was also higher than that of the $T+E_2$ group. However, the average latency

period for the incidence of the T+ DES induced tumors was little bit longer than that of the T+E_2 treatment, as 10 months compared with 8.86 months.

3.2.3 DMBA Induced Mammary Tumors in Female Noble Rats

In comparison, the latency period for mammary tumorigenesis in the carcinogen induction group was relatively short. The first palpable mammary tumor was found in the treated animals only after 2 months of the chemical carcinogenic treatment. The mean latency period for development of mammary tumor was 6.38 months. The overall incidence of breast tumors was as high as 80%.

Figure 3.2.1 showed a comparison of the incidence rate and latency period of mammary tumorigenesis between the carcinogen and hormone treatment in female Noble rats.

3.2.4 NMU Induced Mammary Tumors in Female SD Rats

The incidence rate of NMU-induced mammary tumors in female SD rats was as high as 100%. The mean latency period for the mammary tumorigenesis was 6 months. Generally, more than one mammary tumor could be found from the treated animals. These tumors might be collected form the cervical, thoracic, abdominal or inguinal mammary glands of the animals. No particular tendency for the localization of the induced breast tumors was observed.

3.3 Histology of Normal and Lactating Mammary Glands in Female Noble Rats

Mammary glands in sexually mature virgin Noble rat were characterized by a sparse distribution of terminal end buds, alveolar buds and ductal systems in a large amount of adipose tissues (Figure 3.3.1). The club-shaped terminal buds were lined by a thick epithelium composed of five to ten layers of cells (Figure 3.3.1A). The

alveolar buds were small in size and appeared as clusters of alveolar tubules. The tubules only possessed narrow lumen (Figure 3.3.1B). The mammary ducts also had small lumen. The ductal epithelium was lined by cuboidal cells of one layer thick and was embedded in a small amount of connective tissue (Figure 3.3.1C). Due to their large size, the epithelial tubules were very easy to discover in the stromal tissue.

In the lactating mammary glands, both the terminal end buds and alveolar buds decreased in number significantly (Figure 3.3.2). It was due to the differentiation of the terminal buds into alveolar buds, which further differentiated into lobules. The lactating gland is fundamentally composed of the lobular structures. The lobular structures were large in size. They were composed of increased number of individual alveolar structures. The alveolar structures possessed wide lumens (Figure 3.3.3).

3.4 Histopathology of Mammary Tumors

3.4.1 Histopathology of Spontaneously Developed Mammary Tumors in Noble Rats

Of the 26 spontaneously developed mammary tumors collected from 17 female Noble rats (Table 3.1), histopathological examinations revealed that most of them (80.77%) are benign neoplasm. In the male Noble rat, only one sample of benign fibroadenoma was collected.

The vast majority of the spontaneous tumors collected from the Noble rat colony were fibroadenoma, which comprise 70.37% of all the mammary neoplasms discovered. The malignant carinoma only comprises of 18.2% of all the spontaneous mammary tumor collected from Noble rats.

Mammary areas nearby the tumor were also collected for histopathological examination. Preneoplastic changes were often encountered in these areas, ranging from lobular hyperplasia to atypical hyperplasia. The early preneoplastic change in mammary gland, namely lobular hyperplasia, was characterized by the lobule enlargement (Figure 3.4.1 to 3.4.3). The enlarged lobules were consisted of relatively normal alveoli. The alveoli increased in both number and size. In some cases, the alveoli were filled with proteinaceous secretion that might contain lipid droplets. Cystic changes were also observed (Figure 3.4.8). The alveolar and ductal systems were dilated. The distended lumens were filled with granular materials composed of lipids and proteinaceous materials. On the other hand, the late preneoplastic changes in the mammary gland were characterized by the incidence of atypical hyperplasia. Focal irregular proliferations of epithelium were observed within the mammary ducts or alveoli (Figure 3.4.4 to 3.4.7; Figure 3.4.9 & 3.4.10). The focal proliferation may be appeared in the form of papillary infolding, arches, solid nests or plagues which extended into the lumen from the epithelial layer.

The commonly encountered spontaneously developed fibroademona in Noble rats were composed of both connective tissues and mammary epithelial cells (Figure 3.4.11 to 3.4.16). The proportion of these two cells types varied from sample to samples. In some case, the fibroadenoma was composed almost entirely of connective tissue. However, occasionally, the tumor was mainly epithelial. The histopathology of the fibroadenoma also varied in the secretory behaviors. In some cases, the alveolar lumina were dilated and contained secretory materials. Lipid vacuoles might also present in the epithelium cytoplasm. Nevertheless, in other case, the ductular epithelium was attenuated or atropic. No secretory material was found in the narrow ductal or alveolar lumens.

The histopathology of the fibroadenoma was highly heterogenous. The same tumor might show deviated histopathology. The histopathologicl heterogeneity was observed even in the identical microscopic field (3.4.15).

The other rare benign neoplasms that were collected from female Noble rats included pure fibromas and adenomas. The spontaneously developed fibroma consisted entirely of collagen fibers (Figure 3.4.17). Fibroblasts, arranged in interlacing bundles, were occasionally embedded in the collagen fibers (Figure 3.4.18). Pure adenoma was another rarely encountered spontaneously developed benign neoplasm in Noble rats. The tumor was composed entirely of glandular epithelial structures with a scant connective tissue stroma. The alveoli and ductules were widely distended by proteinaceous secretory material. Cytoplasmic vacuolization was also observed in the alveolar epithelial cells (Figure 3.4.19 & 3.4.20).

In some neoplastic samples developed naturally in Noble rats, there were areas that the alveolar or ductal lumens were partially or completely filled by the tumor cells. These were referred as carcinoma *in situ*. Figure 3.4.21 to Figure 3.4.26 showed some examples of the carcinoma *in situ*, ranging from papillary, cribriform or comedo patterns, which might occur alone or in combination in the neoplastic areas.

In the papillary carcinoma, the ductal structures were dilated and the lining epithelium grew inward to form epithelial papillae. The papillae possessed thin fibrovascular core (Figure 3.4.21 & 3.4.22). Occasionally, the papillae might possess cystic properties (Figure 3.4.23 & 3.4.24). In such cases, the papillae were observed projecting towards the cystic cavity. The fibroconnective tissue in the papillary core was edematous and was infiltrated by lymphoytes. Cell detritus could also be found

in the edematous core. On the other hand, the cribriform pattern was characterized by neoplastic cells which extended into the ductal lumen to from bridges of anastomosing arcades with many vacuole-like structures (Figure 3.4.25). Comedo carcinomas were lesions appeared as distended ductal structures lined by a multilayered epithelium surrounding central necrotic debris (Figure 3.4.26).

As the tumors continued to develop, they invaded the stroma, forming the infiltrating ductal or lobular carcinoma. The infiltrating ductal carcinoma could be developed from the papillary, cribriform or comedo carcinoma *in situ* (Figure 3.4.30; 3.4.32; 3.4.34 to 3.4.40). A sample of infiltrating tubular carcinoma was also collected from the Noble rats. Figure 3.4.27 to 3.4.32 showed the histopathological comparisons between the tubular carcinoma and papillary carcinoma. The tubular pattern was observed as lesion consisted of closely packed and elongated tubular structures (Figure 3.4.27). The lumina of the tubular structures were generally empty and narrow (Figure 3.4.29). Occasionally, secretions were observed in the alveolar lumina (Figure 3.4.31).

Figure 3.4.33 to figure 3.4.38 demonstrated some examples of the infiltrating ductal carcinoma of the cribriform, comedo and papillary patterns. These tumors were still well-differentiated and all retained the tendency to form tubules and alveoli. On the other hand, figure 3.4.39 to figure 3.4.44 illustrated examples of the poorly differentiated carcinomas. These tumors were characterized by areas of poor cellular organizations virtually devoid of glandular formation. These carcinoma cells commonly appeared as isolated islets infiltrating the surrounding connective tissues, adipose tissues or skeletal muscles. In these situations, the tumor was called an anaplastic carcinoma. Figure 3.4.47 showed a histopathological transition of a carcinoma from a relatively well-differentiated type into an anaplastic carcinoma.

In some cases, the incidence of spontaneous mammary tumors in Noble rat was associated with the abnormalities of the other peripheral organ, including liver and lung. Grossly, the abnormality of lung was appeared as the presence of obvious white patches on the surface of the organs. Figure 3.4.45 & 3.4.46 showed a histological examination of the apparently abnormal lung tissues. The alveolar lining was clearly thickened. This abnormal thickening might be due to the deposits of metastastic cancer cells from the mammary origins.

The histopathology of the spontaneously developed mammary tumors in Noble rats was highly heterogenous. The tumor histopathology often differed from portion to portion within the same tumor, and it was not uncommon that more than one histopathological type could be found within the same microscopic field (Figure 3.4.48 to 3.4.53).

3.4.2 Histopathology of Hormone Induced Mammary Tumors in Female Noble Rats

Both the T+E_2 and T+DES-induced mammary carcinomas gave similar morphological appearances. The early histological changes in the hormone-treated mammary glands were characterized by the formation of enlarged cystic lobules (Figure 3.4.54 & 3.4.55). The histology of Individual alveolus of the lobule was relatively normal. The alveoli were lined by a single layer of low cuboidal epithelium. Their lumens were distended and filled with secretory materials. In some case, vacuolization was observed in the cytoplasm of the alveolar epithelial cells. Stroma surrounding the cystic alveoli was composed of normal adipose cells (3.4.56).

Subsequent hormonal treatment resulted in epithelial hypertrophy and hyperplasia. The contour of the alveolus was irregular. The epithelium tended to

grow inward and fill the cystic lumen (Figure 3.4.56 & 3.4.57). The abnormal intraductal epithelial proliferation finally resulted in the formation of the intraducal carinoma. The carcinoma might be in the pathological classification of the papillary pattern, cribriform pattern or the comedo pattern (Figure 3.4.58 & 3.4.59).

Very often, the hormone-induced carcinoma appeared as clusters of detached neoplastic epithelial cells floating on a distended cystic lumen (Figure 3.4.60 & 3.4.61). The core region of the detached carcinoma islets was usually composed of edematous fibroconnective tissues. Infiltration of lymphocytes was sometimes observed.

3.4.3 Histopathology of DMBA Induced Mammary Tumors in Female Noble Rats

The mammary neoplastic samples induced in female Noble rats by the administration of the carcinogen DMBA were all classified as carcinomas. Carcinoma of papillary, cribriform and comedo patterns were collected. Figure 3.4.62 to 3.4.64 showed some representative photomicrographs of the histopathology found in the DMBA induced mammary tumors. In some areas of the invasive neoplastic samples, anaplastic carcinoma was found. The carcinoma cells appeared as clusters of epithelial cells embedded in the stromal tissues (Figure 3.4.65).

3.4.4 Histopathology of NMU Induced Mammary Tumors in Female SD Rat

Carcinomas of various histopathological types were collected from the NMU-treated SD rats. The carcinomas ranged from papillary, cribriform to comedo patterns. These histopathological types might appear alone or in combinations. Occasionally, anaplastic carcinoma was also observed.

3.5 Whole Mount Preparation of Mammary Glands under Hormonal Treatments

Whole mount preparation of virgin female Noble mammary gland was characterized by numerous sparsely distributed lateral alveolar buds on a long narrow mammary duct. Terminal end buds could be found at the end of the terminal ducts (Figure 3.5.1). Both the lateral and terminal buds were small in size. Only low degree of differentiation was observed.

10 days after the simultaneous testosterone and 17-β estradiol treatment, high degree of lobuloalveolar differentiation was observed (Figure 3.5.2). There was prominent lobule formation. The lateral alveolar buds increased significantly in both the number and size. They were often cyst filled. The mammary ducts were also thickened. The terminal end buds were no longer found on the ending of the terminal mammary ducts due to its differentiation to form alveolar buds.

2 months post the combined hormonal treatments, further lobuloalveolar differentiation was observed (Figure 3.5.3). The lateral alveolar buds increased further in number and size. Large cystic buds were frequently encountered in the whole mount specimens.

3.6 Effects of Bilateral Ovariectomy on the Growth of Spontaneously Developed Mammary Tumors

Bilateral ovariectomy was carried out on three individual Noble rats bearing palpable spontaneously mammary tumors. Table 3.5 showed the changes in the volume of the spontaneously developed mammary tumors in the bilateral ovariectomized Noble rats.

Figure 3.5 demonstrated the growth rate of the spontaneously developed mammary tumors after bilateral ovariectomy. The alternations in the hormonal environment by surgical removal of sexual gland generally accompanied by the initial retarded growth of the mammary tumors. Thereafter the mammary tumors continued to grow. Regression of the spontaneously developed mammary tumors was not observed after bilateral ovariectomy.

3.7 Transplanability of the Spontaneously Developed Mammary Tumors in Noble Rats

In order to establish a transplantable tumor line, some of the spontaneously developed mammary tumors collected from the female or male Noble rats were transplanted to other individual rats of the same species and sex. Table 3.6 showed the details of the transplanted spontaneously developed mammary tumors.

Histopathological examinations revealed that all the transplanted tumors were benign fibrodenomas (Figure 3.7.1 to 3.7.3). They were all being successfully transplanted to the host Noble rats. Generally, the tumor transplants needed 4 months' time to become a palpable mass in the host animals. The tumors were then transplanted to other Noble rats to generate subsequent generation of transplanted tumor line. So far, the transplanted tumor lines were in the third generations.

3.8 Examination of the Malignancy of Mammary Tumors by Immunohistochemical analysis of Epithelial Keratin Expression

To investigate the invasiveness of the mammary tumors into the adjacent stromal tissue, immunohistochemisty of a broad spectrum of keratin was carried out.

Some representative photomicrographs were selected to demonstrate the keratin expression in the epithelial cells of the normal Noble mammary gland and spontaneously developed mammary neopalsms (Figure 3.8.1 to 3.8.8).

The cytoplasm of the normal mammary epithelial cells was positively stained for keratins (Figure 3.8.1). The strong cytoplasmic expression was reserved in both the benign neoplasim and carcinoma *in situ* (Figure 3.8.2 to 3.8.4). Figure 3.8.5 to 3.8.8 showed some examples of the highly malignant mammary carcinoma. Clusters of epithelial cells positively stained with cytokeratin were observed invading the surrounding stromal tissues.

3.9 Immunohistochemical Analysis of Expression and Localization of Hormone Receptor Protein in Normal and Neoplastic Mammary Tissues of Female Noble Rats

3.9.1 Expression and Localization of Hormone Receptors in Control Tissue

Uteri samples were collected from adult virgin Noble rat and used as a positive control tissue for the estrogen receptor α (ERα), estrogen receptor β (ERβ) and progesterone receptor (PR). The specificity of the staining was established by omitting the primary or secondary antibody. Figure 3.9.1 showed a negative control sample from a uterus section. In the control sample, primary antibody was omitted and no immunoreactivity was detected.

Figure 3.9.3 to 3.9.8 showed some Noble rat uterine samples stained for the ERα (Figure 3.9.3 & 3.9.4), ERβ (Figure 3.9.5 & 3.9.6) and PR (Figure 3.9.7 & 3.9.8). For the ERα and ERβ, strong nuclear staining was observed in most endometrial and stromal cells. Strong nuclear ERβ staining was also observed in the muscle layers. For the PR, strong nuclear staining was observed in endometrial cells.

However, the stromal and muscle cells only exhibited a moderate level of staining. These results matched well to the published data (Wang H *et al.*, 1999).

For the androgen receptor (AR), adult Noble rat prostate was used as a control tissue. In the rat prostate, strong AR immunoreactivity was observed in the cytoplasm and unclei of the glandular epithelail cells (Figure 3.9.28). The specificity of the positive signal was evaluated by omitting the primary antibody on the control samples. The heavy AR staining was no longer observed (Figure 3.9.27).

For the prolactin receptor (PRLR), lactating mammary glands and livers of female Noble rat were used as the control tissues. In the lactating mammary glands, strong PRLR expression was detected in the cell membranes of the alveolar epithelial cells. Sometimes, moderate cytoplamic PRLR immunostaining was also observed in the epithelium (Figure 3.9.30). In the liver tissues, PRLR immunoreactivity was localized in cell membranes and cytoplasmic granules of the hepatocytes (Figure 3.9.32). The specificity of the immunostaining was tested by omitting the primary antibody on the control tissues. No significant immunoreactivity was detected in both livers and lactating mammary glands of the negative control staining (Figure 3.9.29 & 3.9.31).

Table 3.7 showed a summary of the immunohistochemical analysis of the expression intensities in various hormone receptor proteins in normal and neoplastic mammary tissues in female Noble rats.

3.9.2 Expression and Localization of Estrogen Receptor α

Immunohistochemical staining for ERα in normal female mammary glands revealed that most ductal and alveolar epithelial cells had strong nuclear staining. Figure 3.9.9 showed a terminal end buds heavily stained for the ERα in the nuclei of

most epithelial cells. When the primary antibody was omitted, no immunoreactivity was observed (Figure 3.9.2).

Mammary tumors of different experimental groups also showed a positive nuclear immunoreactivity for ERα. However, a derivation of the expression intensity was observed. In the spontaneously developed mammary tumors, a consistent strong nuclear staining was observed in both benign (Figure 3.9.10) and malignant neoplasms (Figure 3.9.11). In the DMBA-induced mammary tumors, only a weak to moderate nuclear immunostaining was observed (Figure 3.9.12). On the other hand, the hormone-induced mammary tumors, either from T+E_2 (Figure 3.9.13) or T+DES treatment (Figure 3.9.14), exhibited a moderate to strong nuclear immunoreactivity.

3.9.3 Expression and Localization of Estrogen Receptor β

Immunohistochemical analysis of ERβ in normal female mammary gland revealed that a moderate cytoplasmic and nuclear staining was observed in most ductal or alveolar epithelial cells (Figure 3.9.15). In the mammary tumors, the expression of ERβ is varied (Figure 3.9.16 to 3.9.20). In the spontaneously developed mammary tumors, weak cytoplasmic staining was observed in the benign neoplasm (Figure 3.9.16). However, in the malignant carcinoma, a moderate cytoplasmic immunoreactivity was detected (Figure 3.9.17). In contrast, mammary tumors induced by DMBA only exhibited a very weak ERβ immunostaining (Figure 3.9.18). For the hormone induced mammary tumors, a strong cytoplasmic ERβ staining was observed in both the T+E_2 (Figure 3.9.19) and T+DES (Figure 3.9.20) induced mammary carcinomas.

3.9.4 Expression and Localization of Progesterone Receptor

Immunohistochemisty of PR showed that the normal mammary ductal and alveolar epithelial cells only exhibited a moderate nuclear and cytoplasmic

immunoreactivity (Figure 3.9.21). In the spontaneously developed mammary tumors, a strong nuclear PR staining was observed in both benign tumors (Figure 3.9.22) and malignant carcinomas (Figure 3.9.23). On the other hand, moderate to strong nuclear immunoreactivity was also observed in DMBA-induced mammary carcinomas (Figure 3.9.24). In the T+E_2 induced mammary carcinoma, a moderate cytoplasmic and nuclear PR staining was observed (Figure 3.9.25). However, mammary carcinoma induced with T+DES exhibited a strong nuclear staining (Figure 3.9.26).

3.9.5 Expression and Localization of Androgen Receptor

Immunohistochemical staining for AR in normal female mammary glands revealed that most ductal and alveolar epithelial cells only showed a moderate cytoplasmic immunostaining to AR (Figure 3.9.33). In contrast, more intense AR immunoreactivity was observed in the mammary tumors. In the spontaneously developed benign mammary tumors, strong cytoplasmic and nuclear staining was observed in the alveolar epithelium (Figure 3.9.34). The heavy nuclear AR staining was reserved in the spontaneously developed malignant neoplasms (Figure 3.9.35). In the DMBA-induced mammary tumors, only moderate AR nuclear immunostaining was observed in the carcinoma cells (Figure 3.9.36). The hormone-induced mammary tumors, either from T+E_2 (Figure 3.9.37) or T+DES treatment (Figure 3.9.38), exhibited a moderate to strong cytoplasmic and nuclear immunoreactivity.

3.9.6 Expression and Localization of Prolactin Receptor

Immunohistochemical analysis of PRLR in normal female mammary gland revealed that only a weak to moderate cytoplasmic staining was detected in the alveolar epithelial cells (Figure 3.9.39). In the spontaneously developed adenomas, strong PRLR immunoreactivity was observed in the plasma membrane and

sometimes in the cytoplasm of the most epithelial cells (Figure 3.9.40). The intense cell membrane and cytoplasmic PRLR immunoreactivity was also detected in the spontaneously developed carcinoma cells (Figure 3.9.41). Similarly, mammary tumors induced by DMBA also exhibited strong PRLR immunostaining in focal areas (Figure 3.9.42). For the T+E_2 induced mammary tumors, only weak to moderate PRLR immunoreactivity was observed (Figure 3.9.43). In contrast, carcinoma induced by T+DES exhibited strong PRLR immunoreactivity (Figure 3.9.44).

3.10 Western Blot Analysis of Expression of Hormone Receptor Proteins in Normal and Neoplastic Mammary Tissues of Female Noble Rats

3.10.1 Expression of Estrogen Receptor α

Western Blot analysis of ERα identified several bands on SDS-PAGE at ~67, 54 and 50 kDa in Noble rat uteri (Figure 3.10.1). Mammary samples from untreated female Noble rats exhibited a very low expression of the 67 kDa native ERα protein. The expression of the 67 kDa protein was uplifted in the spontaneously developed benign and malignant mammary tumors. In the spontaneously developed neoplastic samples, an expression of the 54 kDa putative ERα was detected. In the spontaneously developed carcinoma, there was a further expression of the 50kDa putative ERα. In the DMBA induced carcinoma, an expression of the 67 kDa native and 50kDa putative ERα protein was detected. Mammary carcinoma induced by combined hormonal treatment, either T+E_2 or T+DES exhibited an intense

expression of the 67kDa ERα protein. Expression of other putative ERα protein was not detected.

3.10.2 Expression of Estrogen Receptor β

Western blot analysis of ERβ in Noble rat uteri detected an expression of ~55 kDa ERβ protein (Figure 3.10.2). A very low expression level of ERβ was also detected in normal female Noble rat mammary glands. A marked increase of ERβ expression level was observed in the spontaneously developed mammary tumors. In comparison, the spontaneous carcinoma exhibited higher protein expression intensity than the spontaneous benign lesions. In the carcinogen or hormone induced mammary carcinoma, expression of 55kDa ERβ protein was not detected.

3.10.3 Expression of Progesterone Receptor

Western blot analysis of Noble rat uteri detected the presence of several PR isoforms (Figure 3.10.3). In the uteri samples, high expression of ~82kDa PR-A2 and moderate expression of ~98kDa PR-A1 and ~64 kDa PR-C were observed. In normal female Noble mammary gland, the strong expression of PR-A2 remained. A low expression of PR-C isoform was also detected. Benign mammary tumors developed spontaneously in Noble rats exhibited a moderate expression of PR-A2 protein. The expression level of PR-C isoform in the benign lesions was comparable to that of the normal mammary glands. In the spontaneously developed carcinoma, both the expression of PR-A2 and PR-C was obviously elevated. A further expression of the ~116kDa PR-B and ~98 kDa PR-A1 was also observed in the spontaneous carcinoma samples. In other carcinoma samples induced by either DMBA or combined hormonal treatment, there was a consistent high expression of PR-A2 protein. When compared with the spontaneously developed carcinoma, the expression of PR-C protein was reduced. Both the DMBA and $T+E_2$ induced

carcinoma showed a low expression of PR-C protein whereas in the T+DES induced carcinoma, expression of PR-C was not detected.

3.10.4 Expression of Androgen Receptor

AR protein was not detected in western blot analysis of the receptor in female Noble mammary glands (Figure 3.10.4). In the spontaneously developed benign mammary tumors, a weak expression of both AR-A (~97kDa) and AR-B (~116kDa) proteins were detected. A further elevation of the ~97 and ~116 kDa AR protein expression was observed in the spontaneously developed mammary carcinomas. In the DMBA induced mammary carcinoma, only an expression of 97kDa AR-A protein was detected. In the hormone induced mammary carcinomas, the presence of AR-A protein was also observed. However, its expression was much weaker than the carcinogen induced carcinomas. Expression of the AR-B protein was not detected in both the T+E_2 and T+DES induced neoplasms.

3.10.5 Expression of Prolactin Receptor

Noble rat liver was used as a control tissue to evaluate the expression of the long form and short form prolactin receptor protein on the SDS-PAGE. It was known that both receptor isoforms could be found in the liver although the short form represented the dominant species. Western blot analysis of PRLR identified a strong expression of ~40kDa PRLR protein on the liver tissue. This ~40kDa protein was considered as the short form prolactin receptor. Expression of the short PRLR isoform was not detected in the other normal or neoplastic mammary tissues.

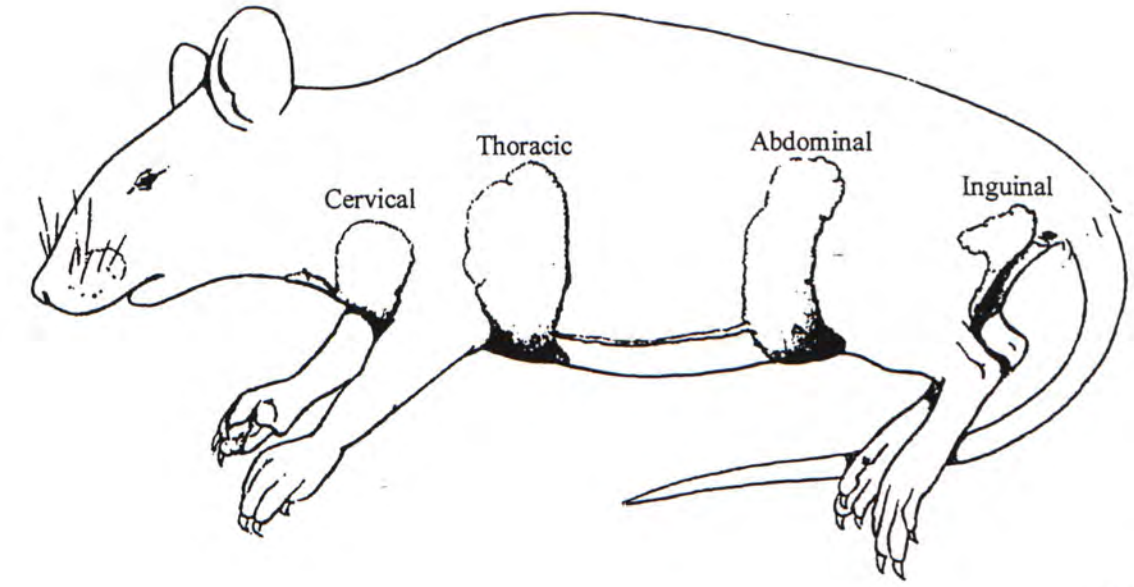
On the other hand, a weak expression of a PRLR protein with molecular size about 65kDa was also detected on the liver tissues. This was regarded as the long PRLR protein. Expressions of the long isoform were detected in all normal and

neoplastic mammary samples, including the spontaneously developed, hormone-induced and carcinogen-induced mammary tumors.

Figure 3.1.1 Schematic Representations of the Locations of the Six Pairs of Rat Mammary Glands.

(A) Ventral View of the Rat Mammary Glands. The rat's mammary glands are distributed in pairs along the milk line, with one pair located in the cervix, two in the thorax, another two in the abdomen and one in the inguinal regions.

(B) Lateral View of the Rat Mammary Gland. The nipples of the mammary gland are medially located and from there the mammary ductal system extends subcutaneously and dorsolaterally into the mammary fat pads.


Cervical
Thoracic
Abdominal
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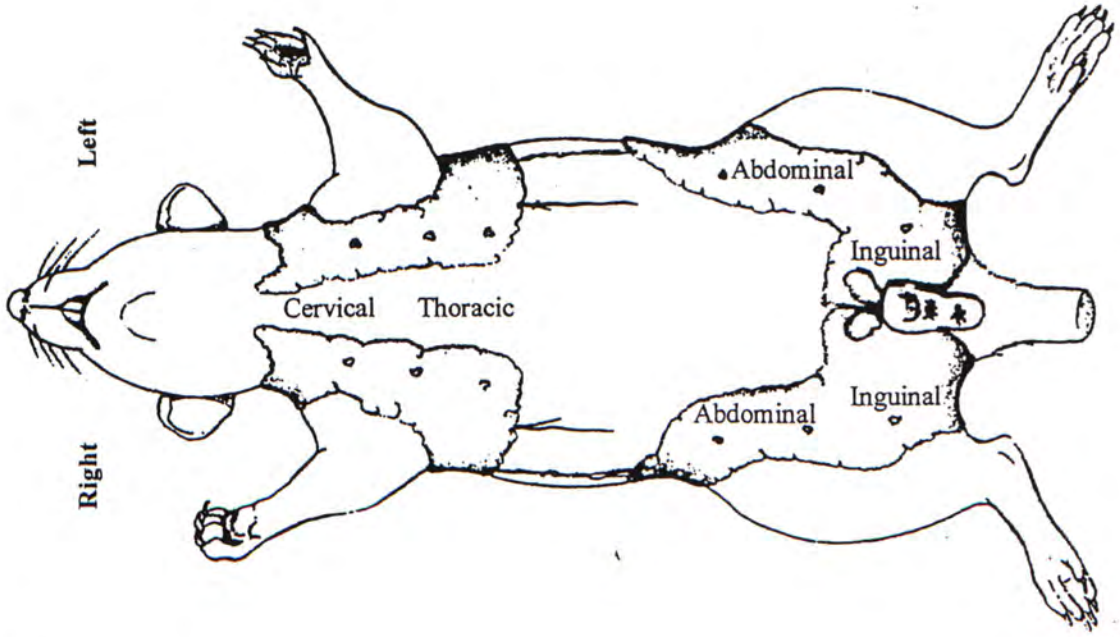
B


Left
Right
Cervical
Thoracic
Abdominal
Inguinal
Abdominal
Inguinal

A

3.1.1

Figure 3.1.2 Gross Appearance of Mammary Tumors Under Different Treatments in Female Noble Rats.

(A) Spontaneously Developed Mammary Tumor. Milk secretions, which appeared in the form of white patches (indicated by arrows), were frequently observed in mammary areas nearby the tumors.

(B) DMBA-Induced Mammary Tumor. Mammary tumor induced by carcinogens usually appeared as a solid mass.

(C) Hormone-Induced Mammary Tumor. Black liquid lesions (indicated by arrows) were commonly found in mammary areas near or far away from the hormone-induced mammary tumors.

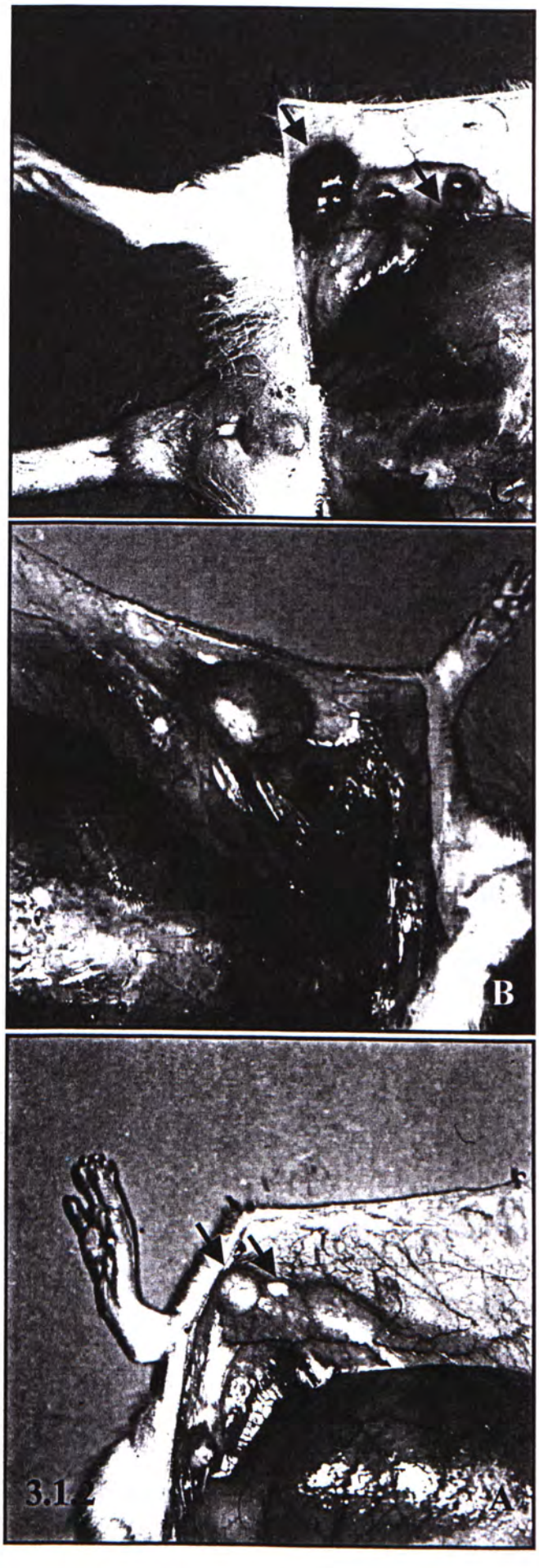

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Figure 3.2.1 Cumulative Incidence of Spontaneously Developed Mammary Tumors in Female Noble Rats

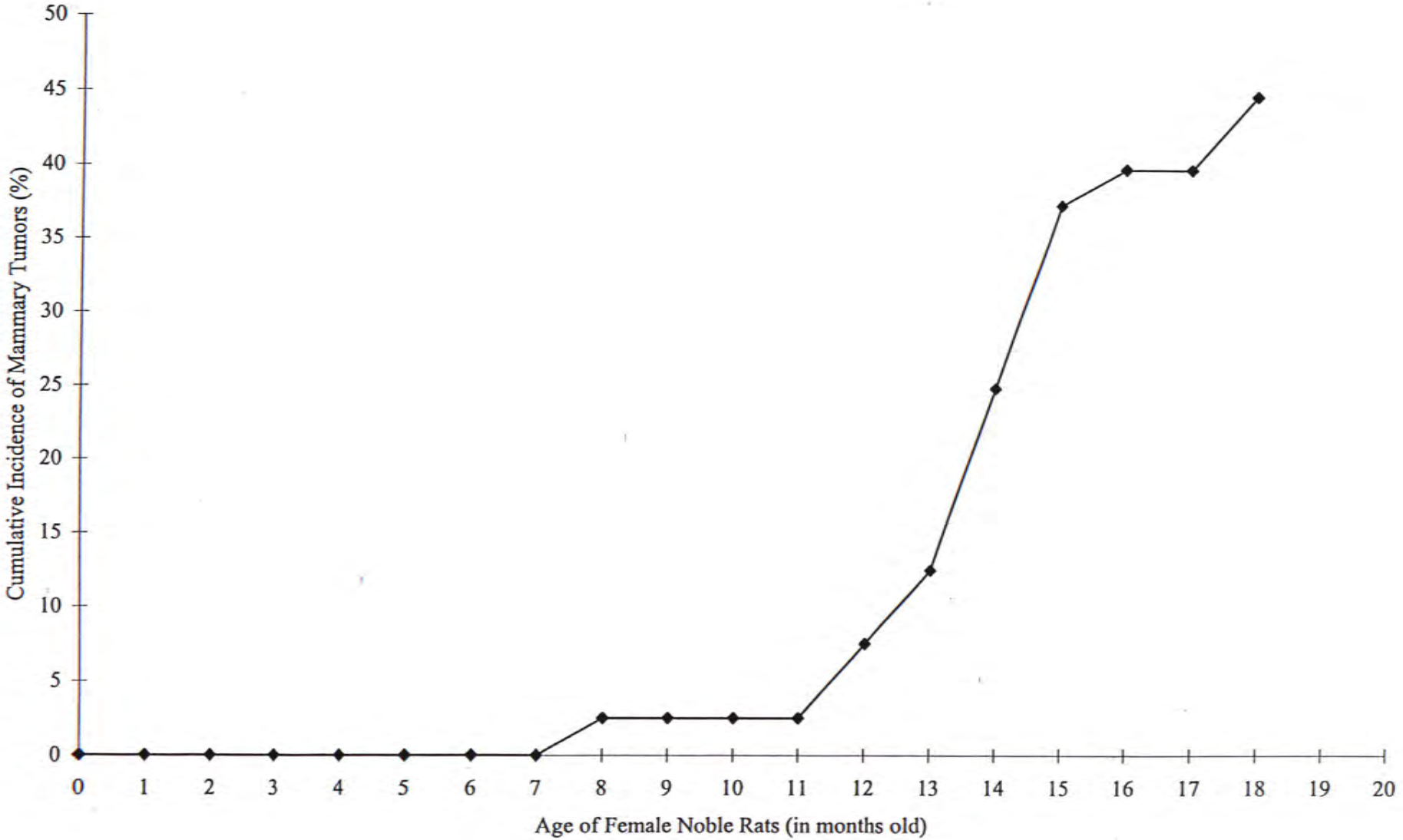


Figure 3.2.2 Cumulative Incidence of Mammary Tumors in the Carcinogen and Hormone-Treated Female Noble Rats

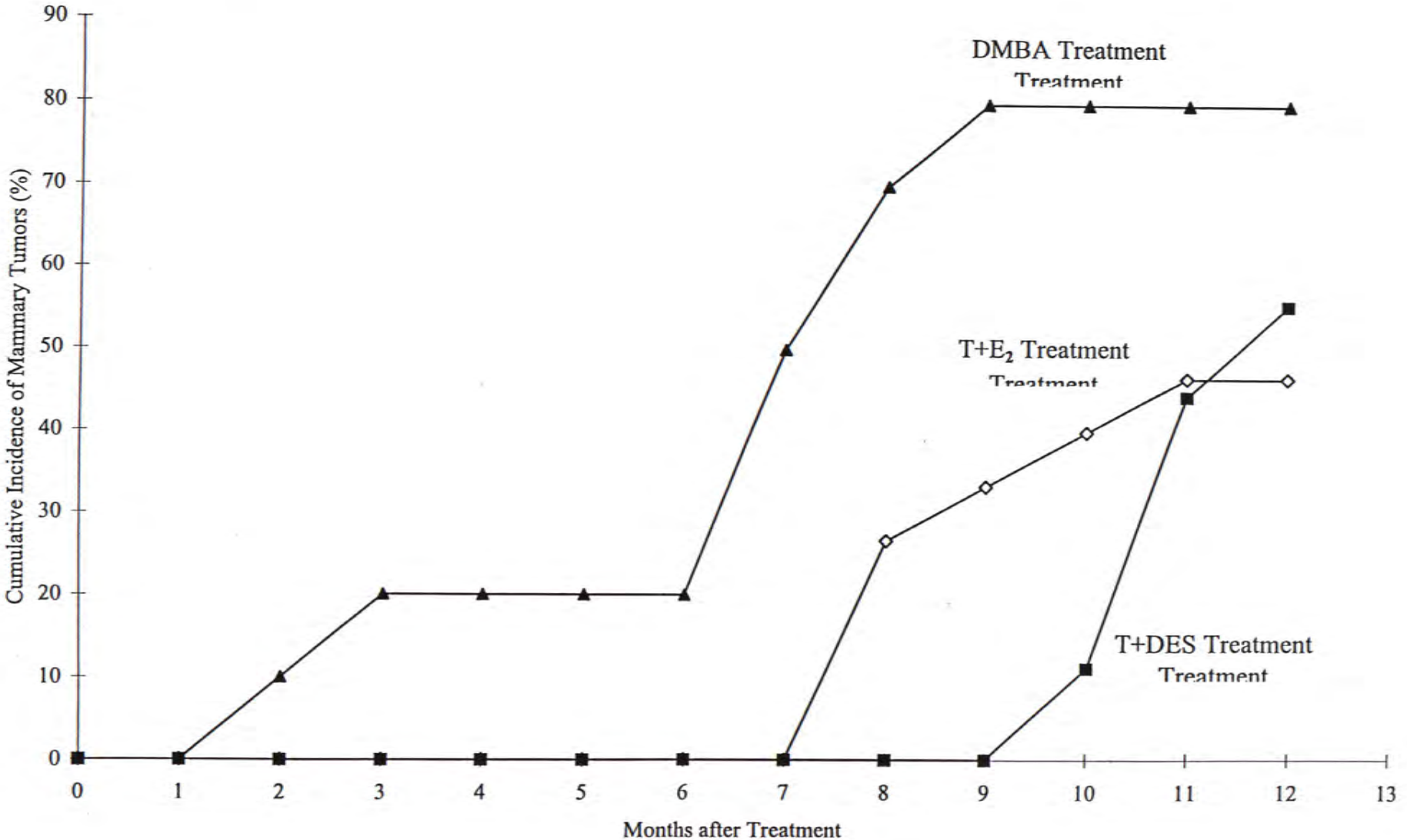
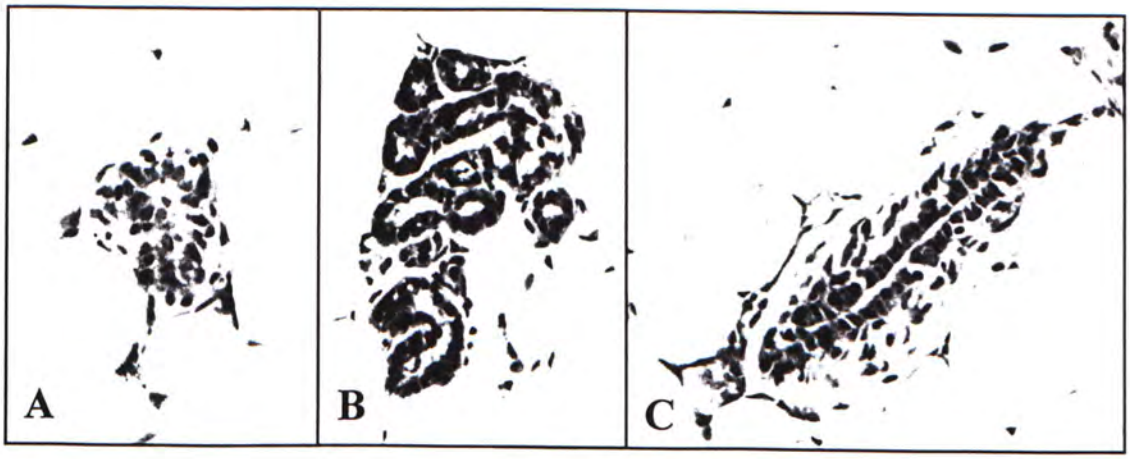


Figure 3.3.1 Histology of Rat Normal Mammary Gland. In adult virgin rats, the inactivated mammary glandular structures were surrounded by large amount of fibrous and adipose tissues. Terminal end buds (TEB), alveolar buds (AB) and ductal structures were sparsely distributed in the stroma.

(A) Terminal End Bud (TEB). TEB was composed of several layers of epithelial cells surrounding a narrow lumen.

(B) Alveolar buds (AB). ABs appeared as clusters of 3-5 tubules, each having a centrally located lumen surrounded by a layer of cuboidal epithelial cells.

(C) Duct. The epithelial component of the duct was composed of an inner luminal epithelial layer surrounded by myoepithelial cells. The duct was separated from the surrounding mesenchymal cells and connective tissues by the basement membrane.


A
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C

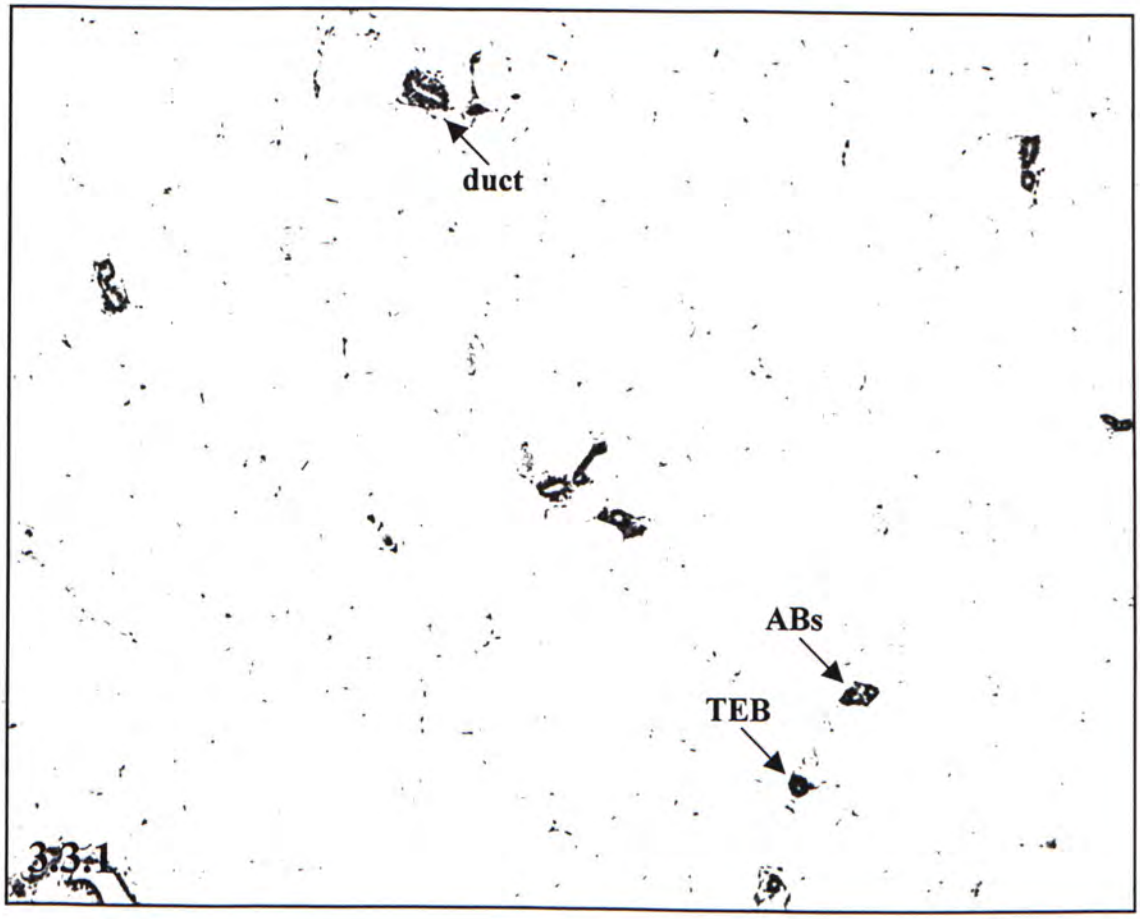
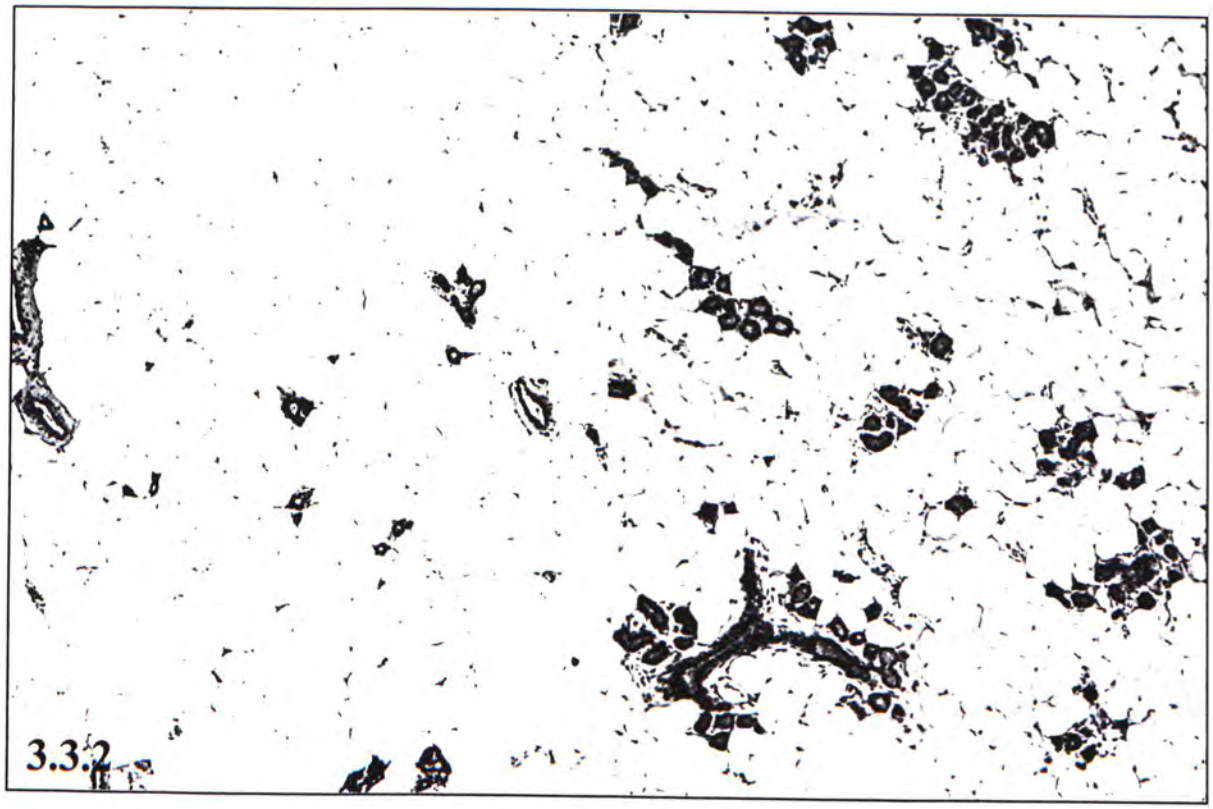

duct
ABs
TEB
3-3-1

Figure 3.3.2 Histology of Female Rat Normal Mammary Gland (left) and Lactating Mammary Gland (right). The glandular area increased significantly in the lactating mammary gland. The lactating gland was composed mainly of the enlarged lobular structures. TEBs fundamentally disappeared due to their differentiation into the lobular structures.

Figure 3.3.3 Higher Magnification of the Lobular Structure in the Virgin Mammary Gland (left) and Lactating Mammary Gland (right). The lobular structures in the lactating gland were enlarged and composed of more and larger individual alveolar structures.



3.3.2

3.3.3

Figure 3.4.1 – 3.4.6 Non-Neoplastic Lesions. The figures showed a series of pre-neoplastic changes of a mammary gland from lobular hyperplasia into atypical hyperplasia.

Figure 3.4.1 Lobular Hyperplasia with Cystic Changes. Lobular hyperplasia consisted of enlarged lobules of relatively normal appearing alveoli. The alveoli increased in number and size, and were usually filled with proteinaceous secretion containing lipid droplets. Large ductal cysts could also be found from the figure.

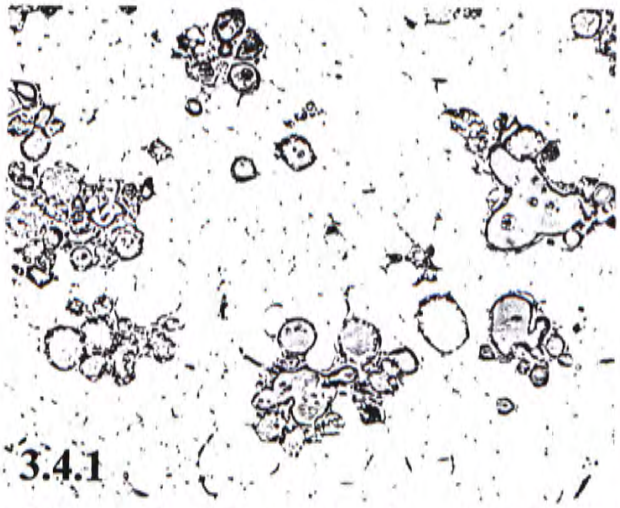
Figure 3.4.2 Severe Lobular Hyperplasia. The gland was mainly composed of enlarged lobule.

Figure 3.4.3 Higher Magnification of Alveoli in Hyperplastic Lobules. The histology of the alveolar epithelial cells was still appearing normal. The alveolar epithelium was one cell thick and cuboidal in shape. Individual alveolus within the lobule was separated by a thin layer of connective tissues.

Figure 3.4.4 Early Atypical Hyperplasia. Atypical hyperplasia consisted of focal irregular epithelium proliferation within ducts or alveoli. The arrow indicated a hyperplasic epithelium extending into the cystic ductal lumen.

Figure 3.4.5 Mild Atypical Hyperplasia. The arrow showed a mild epithelial hyperplasia found in the arch of a ductal cyst. Epithelium besides the hyperplasic cells was still one cell thick.

Figure 3.4.6 Severe Atypical Hyperplasia. The arrow indicated a severe epithelial atypical hyperplasia in a ductal cyst.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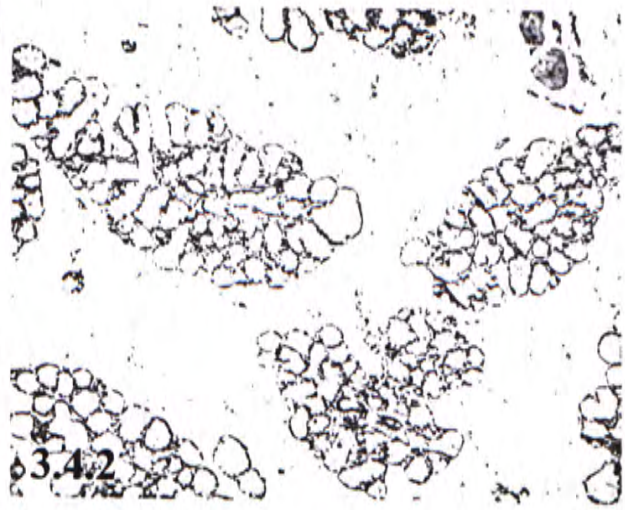
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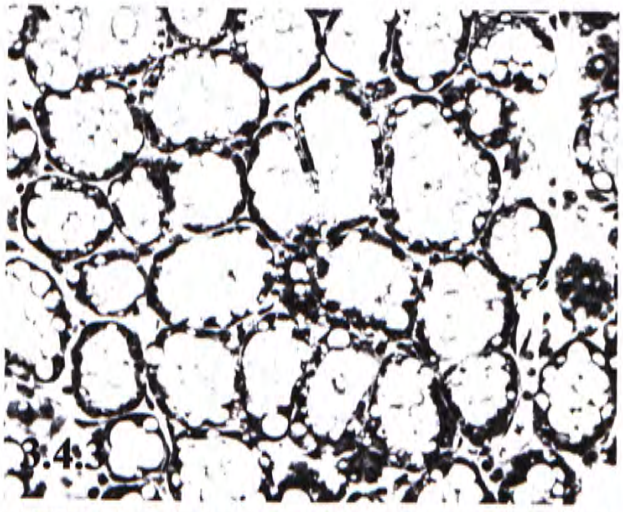
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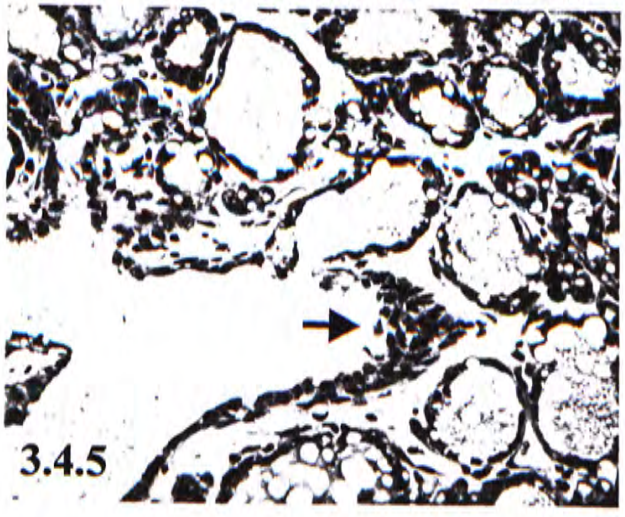
3.4.4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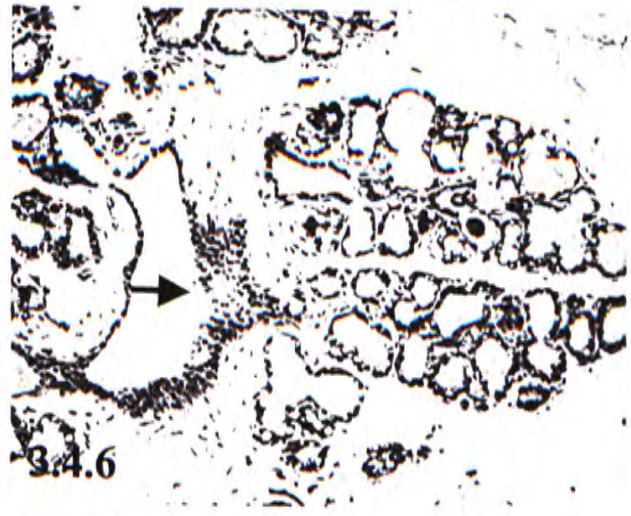

3.4.6

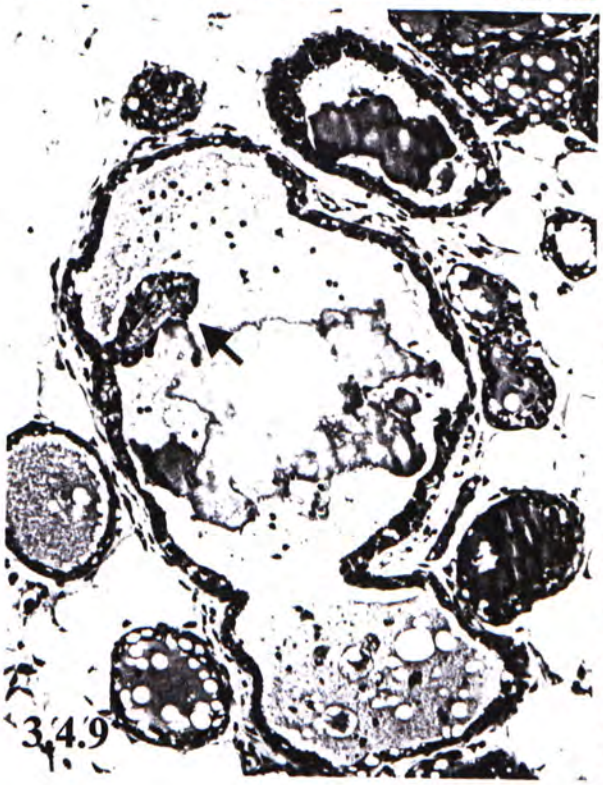
Figure 3.4.7 Lobular Hyperplasia and Atypical hyperplasia. The stomal area was essentially occupied with enlarged lobules. Individual alveolar unit was filled with proteinaceius secretion. Hyperplasic alveolar and ductal epithelium was also found.

Figure 3.4.8 Cysts. Cysts were common non-neoplastic structures found in the mammary gland. They might arise from either ductal or lobular elements. Secretory materials were usually found in the distended cystic lumen.

Figure 3.4.9 & 3.4.10 Atypical Hyperplasia. Hyperplasic epithelial plaques extended into the cystic ductal lumen (black arrows).

3.4.7

3.4.8



3.4.9

3.4.10

Figure 3.4.11 – 3.4.16 Benign Neoplasms.

Figure 3.4.11 Fibroadenoma. The figure showed a fibroadenoma developed spontaneously in an aged male Noble rat. The tumor consisted of concentric layers of dense connective tissue with a small number of widely dispersed ductules.

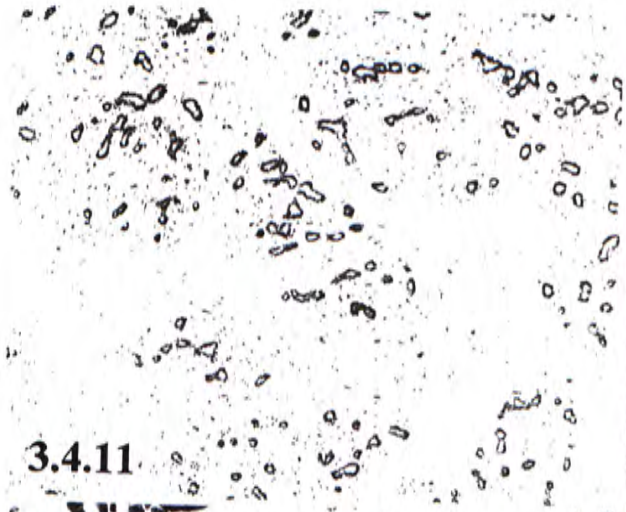
Figure 3.4.12 Fibroadenoma. The figure showed a fibroadenoma collected from an aged female Noble rat. The alveoli within the lobule were secretory. Lipid vacuoles were also observed in the alveolar cytoplasm. Connective tissue distributed within and between the lobules.

Figure 3.4.13 Ductules in Male Noble Fibroadenoma. The ductular epithelium was attenuated and atrophic. Individual ductle was widely separated by concentric layers of connective tissue.

Figure 3.4.14 Secretory Alveolar Epithelium in Female Fibroadenoma. Lipid vacuoles were present in the alveolar epithelium cytoplasm. The alveolar lumina were dilated and contained secretory material.

Figure 3.4.15 Heterogeneity of Mammary Tumor Histopathology. Progressive change in the histopathology of a fibroadenoma from non-seretory tubular pattern into secretory pattern.

Figure 3.4.16 Focal Area of Atypica in Fibroadenoma.



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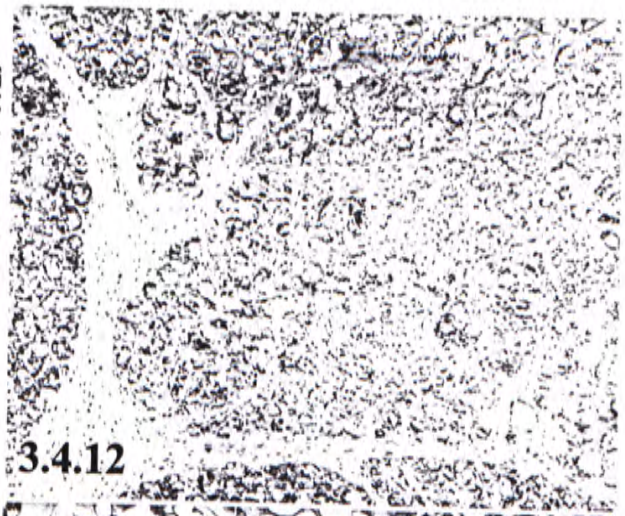

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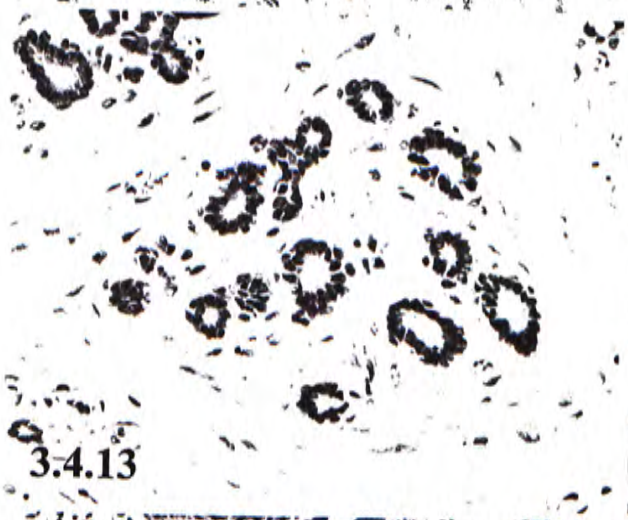

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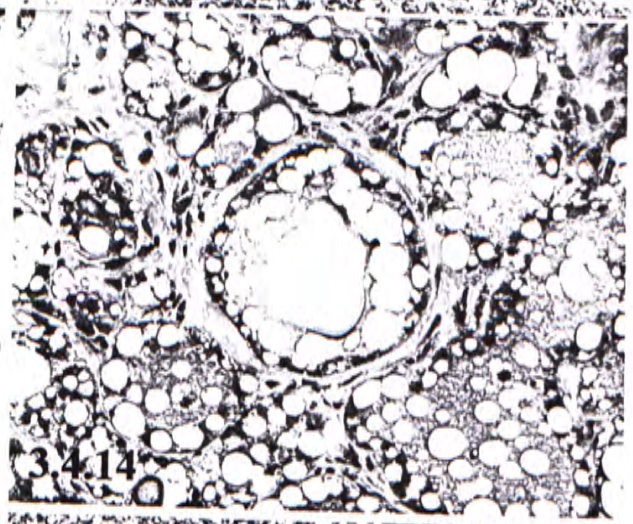

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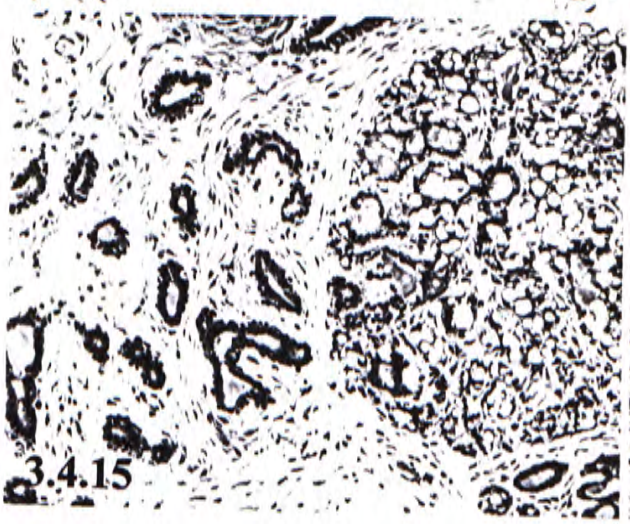

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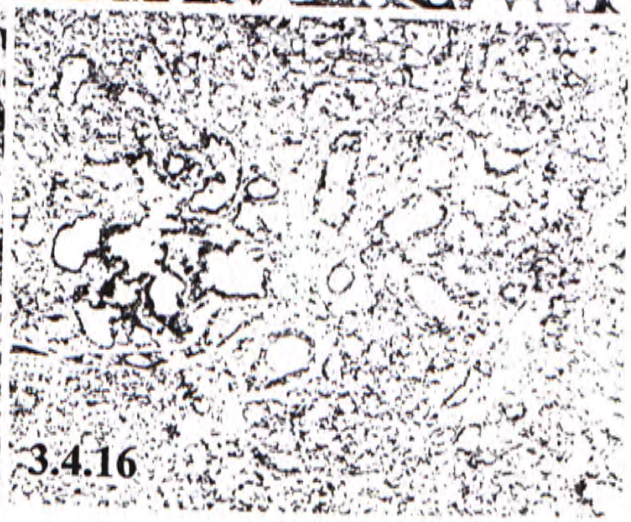

3.4.16

Figure 3.4.17 – 3.4.20 Benign Neoplasms.

Figure 3.4.17 Fibroma. The tumor composed entirely of collagenous connective tissues without any epithelial component.

Figure 3.4.18 Fibroma. The figure showed a fibroma with fibroblastic proliferations.

Figure 3.4.19 Adenoma. The tumor was composed entirely of glandular epithelial structures. Secretory material could be observed in the alveolar lumina and epithelial cytoplasm.

Figure 3.4.20 Higher Magnification of the Alveolar Epithelium in Adenoma.

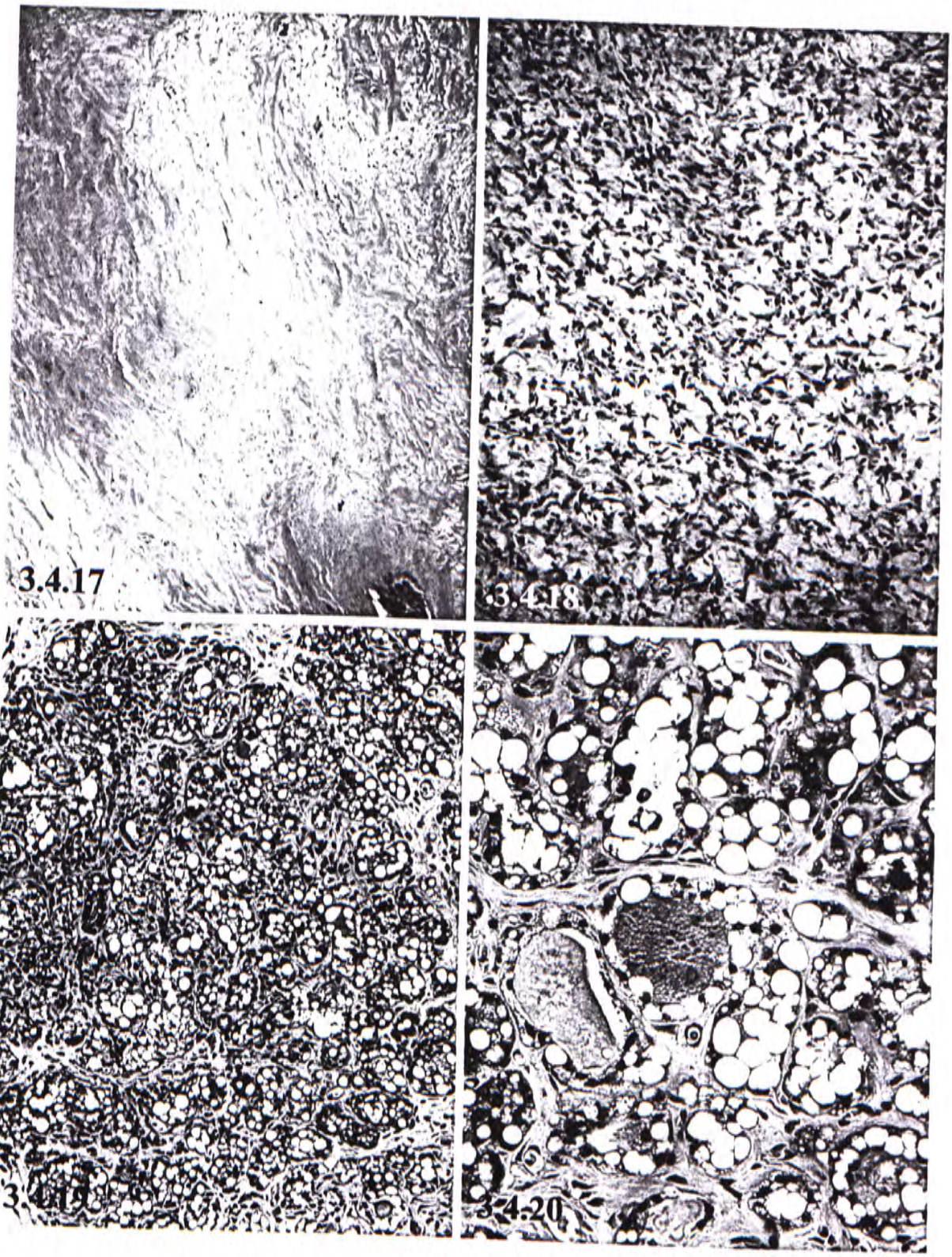
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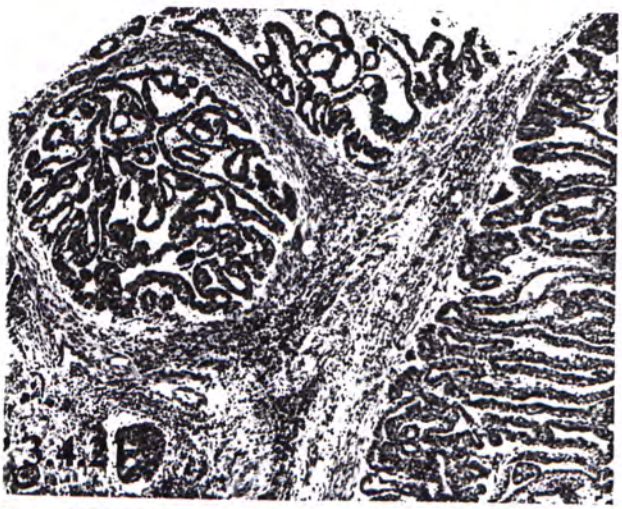
Figure 3.4.21 – 3.4.26 carcinoma *in situ*

Figure 3.4.21 & Figure 3.4.22 Papillary Ductal Carcinoma. The ductal structures were dilated and occupied by the epithelial papillae.

Figure 3.4.23 & Figure 3.4.24 Papillary Ductal Carcinoma with Cystic Structures. Papillae lined by layers of cuboidal epithelial cells projected towards cystic cavity. The fibroconnective tissue in the papillary core was edematous. Figure 3.4.24 showed the details of the papilla. The edematous core of the papilla was infiltrated by lymphocytes and contained cell detritus.

Figure 3.4.25 Cribriform Ductal Carcinoma. Formation of secondary lumina was found in the intraductal epithelium proliferations.

Figure 3.4.26 Comedo Ductal Carcinoma. The carcinoma appeared as a distended ductal structure lined by multilayered epithelium. Necrotic debris was found in the central lumen.



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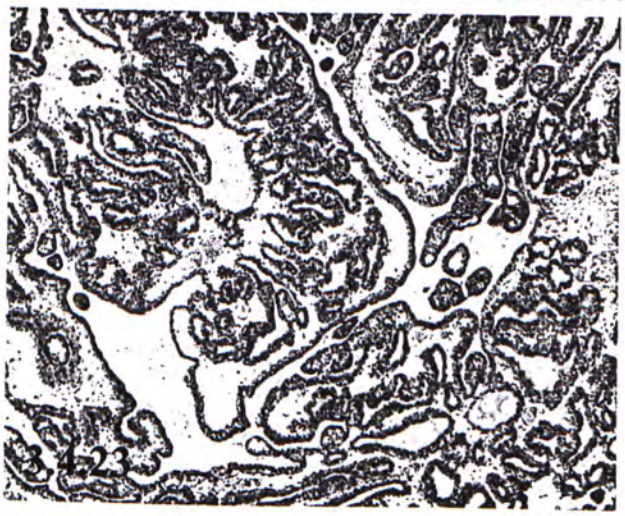

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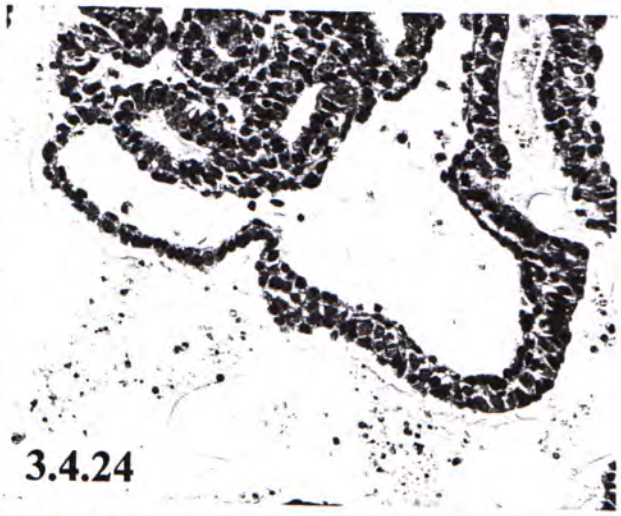

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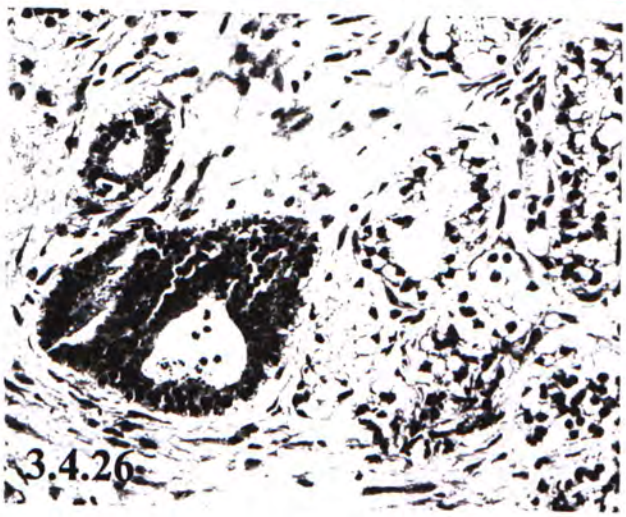

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Figure 3.4.27 – 3.4.32 Comparison Between Tubular Carcinoma and Papillary Carcinoma.

Figure 3.4.27 Tubular Carcinoma. There was a monotonous expense of closely packed tubular structures varying from round to elongated in shape.

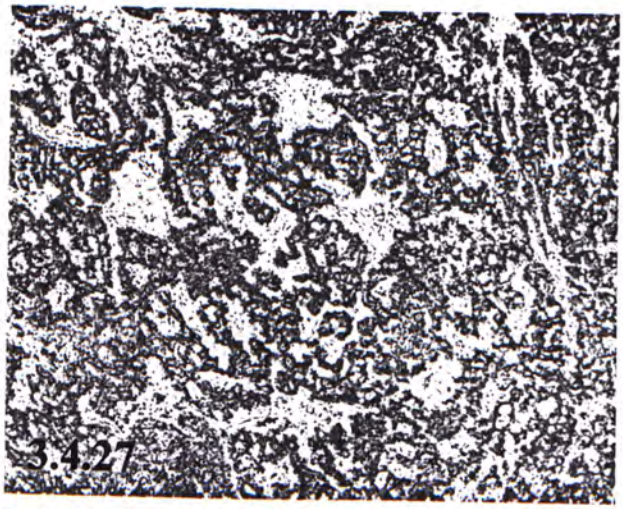
Figure 3.4.28 Papillary Carcinoma. The tumor consisted of multiple branching papillae covered by layers of cuboidal epithelial cells.

Figure 3.4.29 Details of the Tubular Carcinoma. The lumina of the tubular carcinoma were usually narrow and empty.

Figure 3.4.30 Higher Magnification of Papillae in Papillary Carcinoma. The papillae were covered by cuboidal epithelial cells.

Figure 3.4.31 Tubular Carcinoma. The lumina of the tubular structures were generally empty and narrow. Occasionally, secretory material could be found.

Figure 3.4.32 Papillary Carcinoma. The figure showed an adenoma besides a papillary carcinoma.



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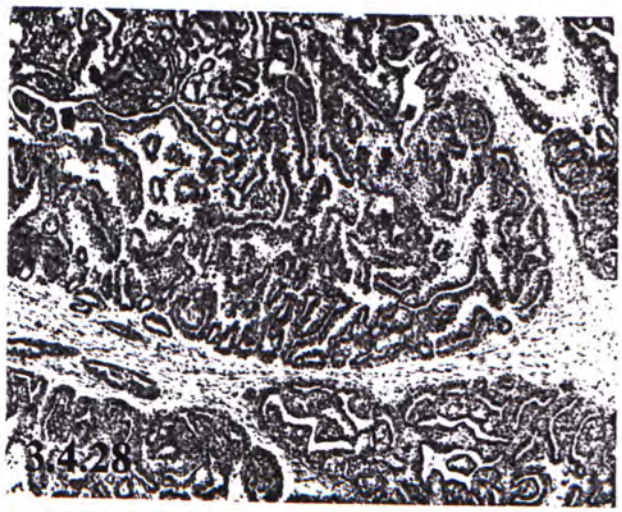

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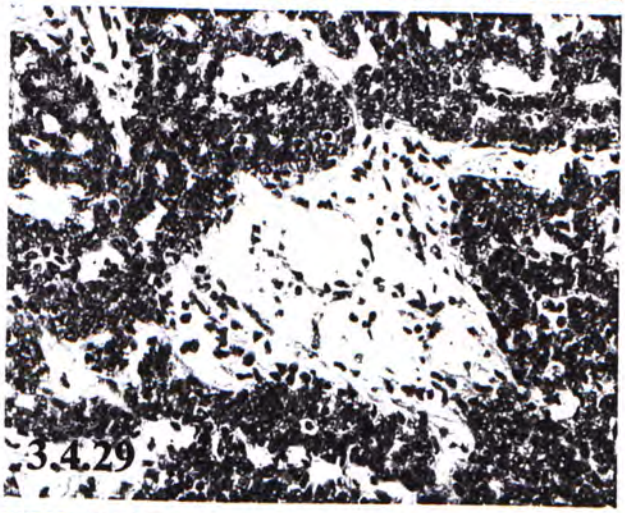

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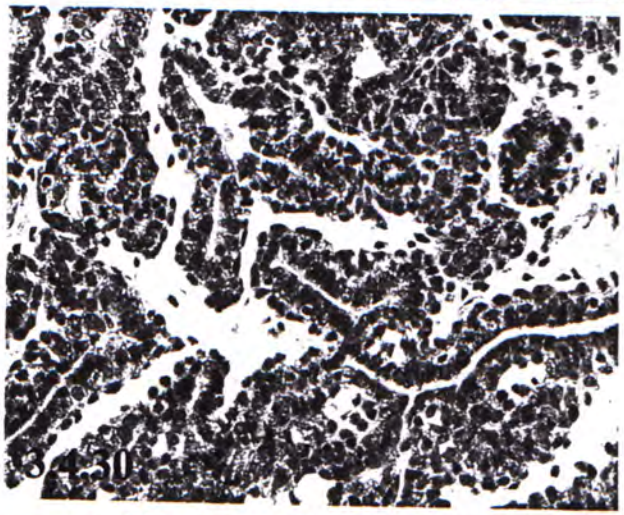

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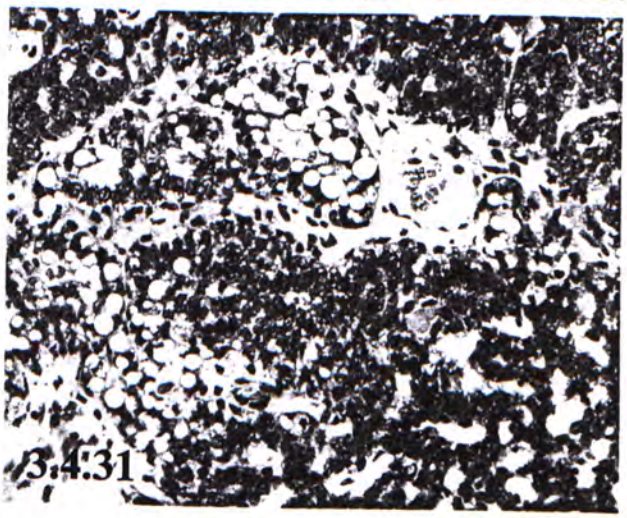

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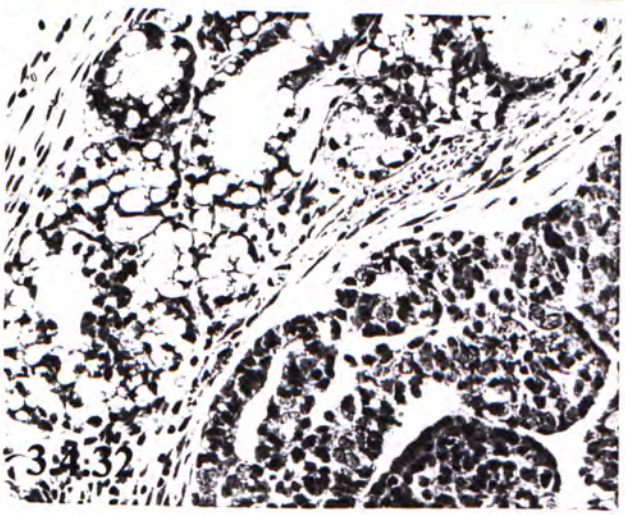

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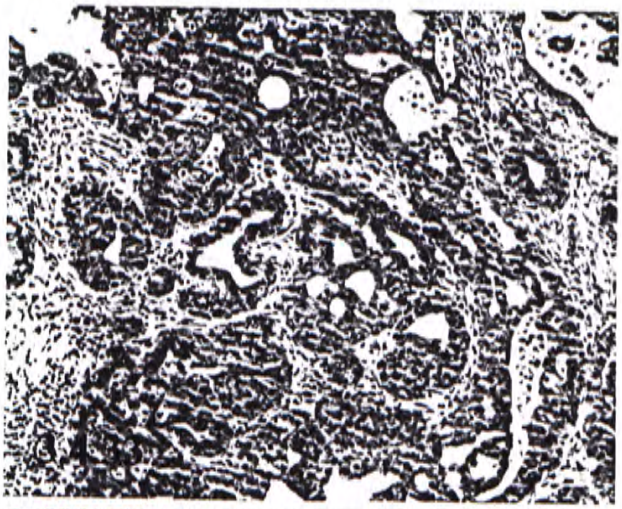
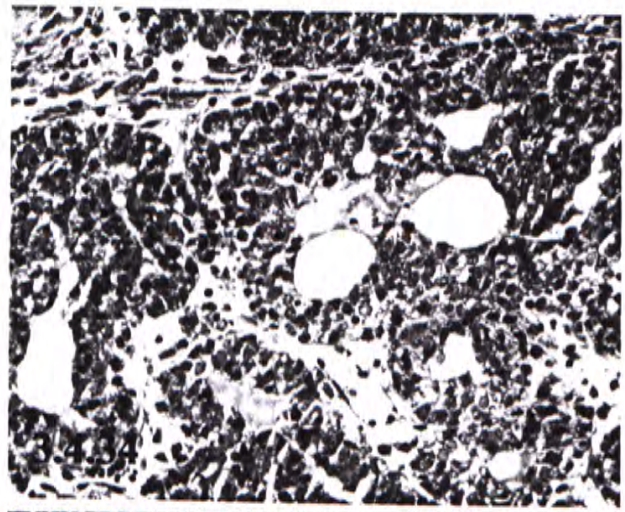
Figure 3.4.33 –3.4.38 Invasive Ductal Carcinoma.

Figure 3.4.33 & Figure 3.4.34 Invasive Cribriform Carcinoma. Secondary lumina were observed in the carcinoma.

Figure 3.4.35 & Figure 3.4.36 Invasive Comedo Carcinoma.

Figure 3.4.37 & Figure 3.4.38 Invasive Papillary Carcinoma.



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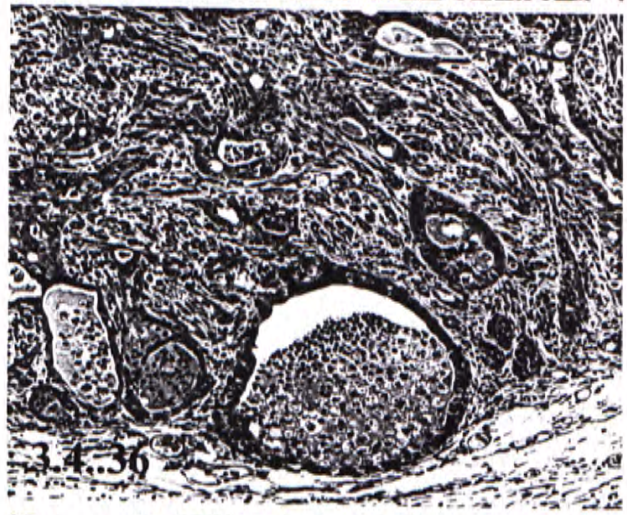
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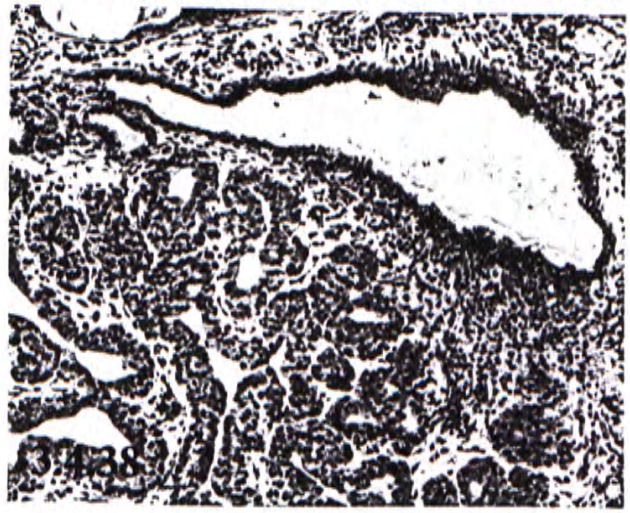
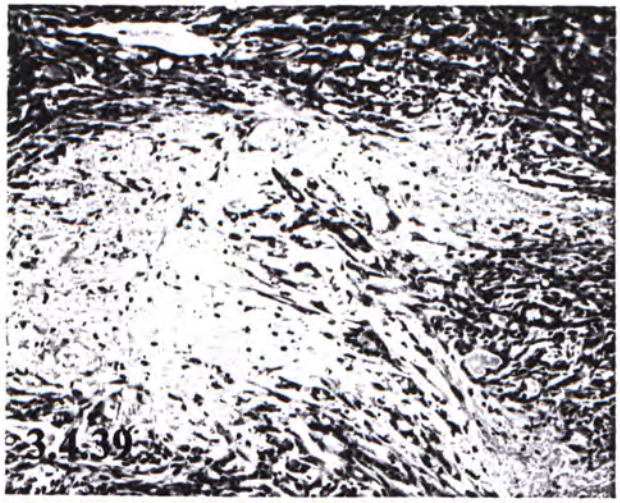


Figure 3.4.39 – 3.4.42 Carcinoma Invasion to Stromal Tissue.

Figure 3.4.43 – 3.4.44 Carcinoma Invasion to Adjacent Skeletal Muscles.

Figure3.4.45 – 3.4.46 Suspected Metastasis of Mammary Carcinoma to Lung. The alveolar lining of lung was obviously thickened. This might due to the deposit of the metastatic mammary carcinoma cells on the alveolar wall.



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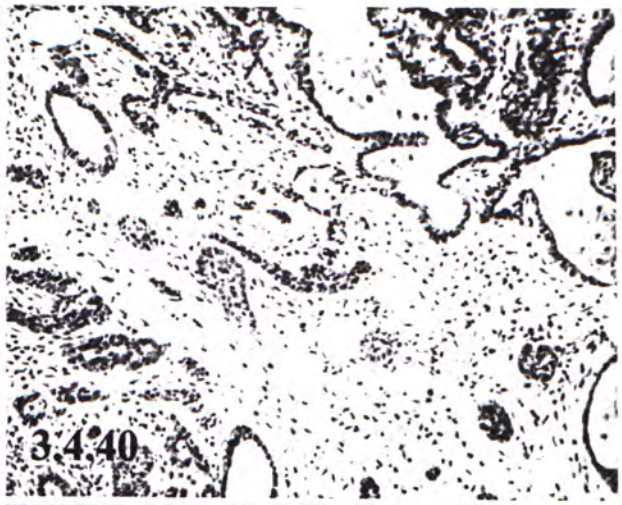

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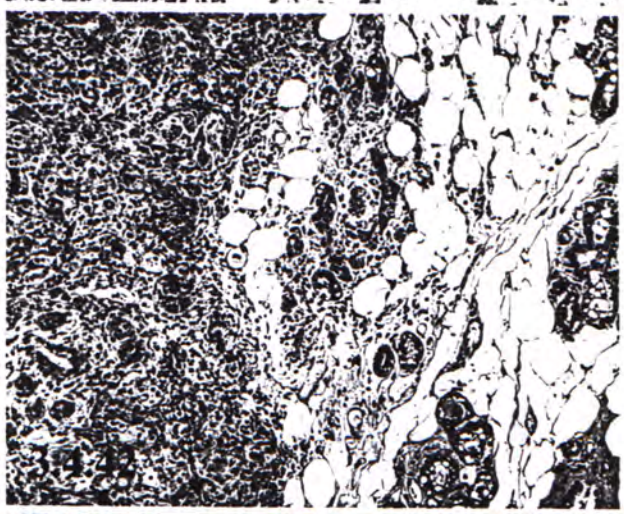

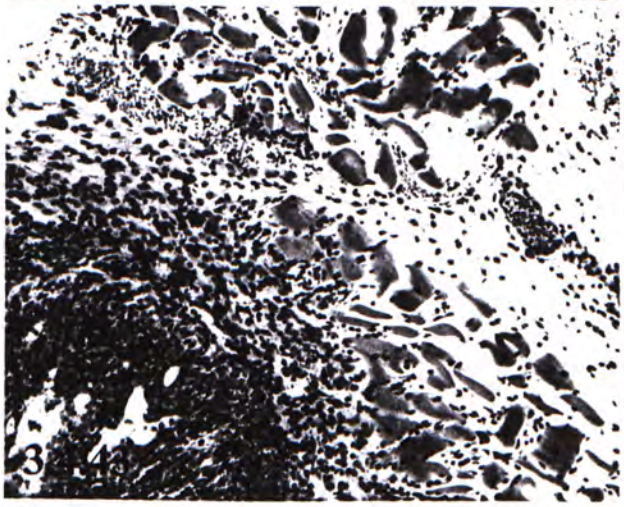

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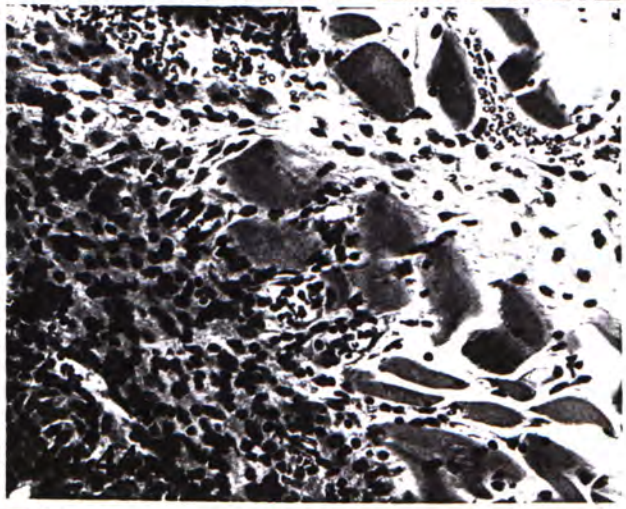

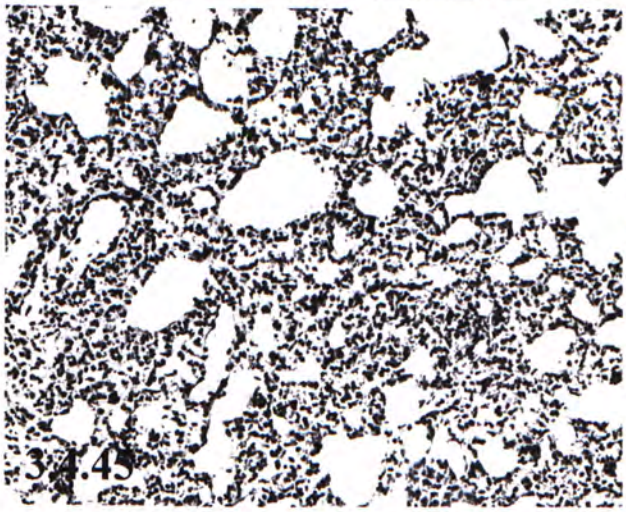

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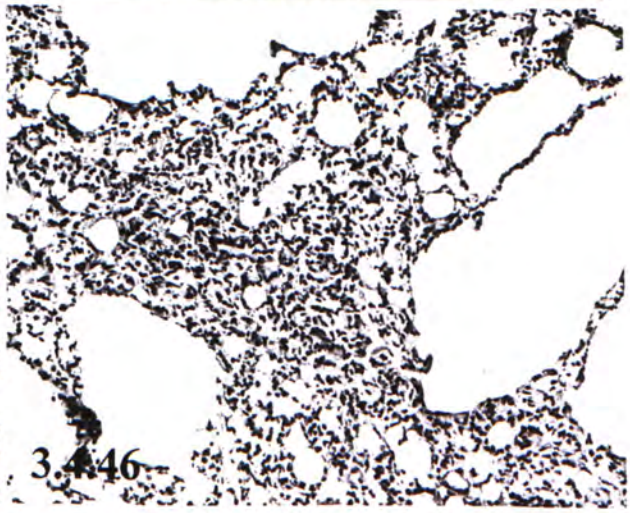


3.4.46

Figure 3.4.47 Invasion. The upper right corner of figure 3.4.47 showed a relatively well-differentiated region in a mammary carcinoma. In this area, the carcinoma cells retained the tendency to form the ductal or alveolar cystic structures. Figure A and B showed the details of these cystic structures. The lower left corner of figure 3.4.47 showed a poorly-differentiated anaplastic carcinoma. Cluster of epithelial cells could be found in the stromal tissues. Figure C and D showed the details of these infiltrating carcinoma cells.

A
B
C
D

Figure 3.4.48 – 3.4.53 Histopathological Heterogeneity of Spontaneously Developed Mammary Tumor.

Figure 3.4.48 There was a progressive increase of fibroblastic proliferation in this sample of fibroma. The upper left corner of the tumor was composed entirely of collagenous connective tissue. In the lower right corner, bundles of fibroblasts were observed.

Figure 3.4.49 The upper left corner of the figure showed a fibroma. However, on the lower left corner, the histopathology of tumor shifted arbitrarily into a secretory fibromadenoma. On the lower right corner, a non-secretory fibroadenoma was observed.

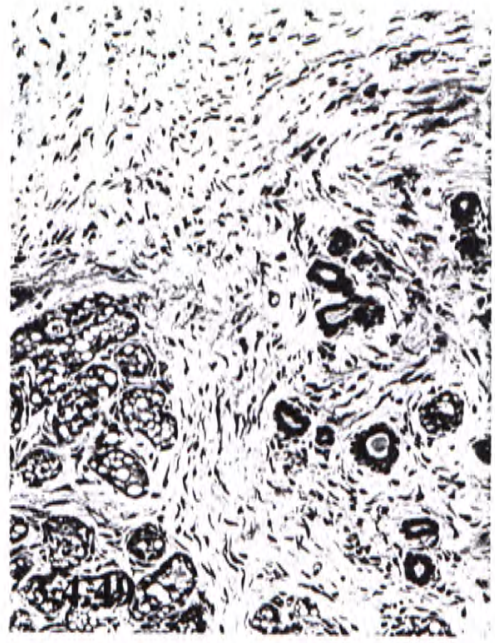
Figure 3.4.50 A focal secretory area was observed in the tubular carcinoma.

Figure 3.4.51 The pathology of the tumor progressively changed from an adenoma (upper region) into a comedo carcinoma (lower region).

Figure 3.4.52 A comedo ductal carcinoma was found in a fibroadenoma.

Figure 3.4.53 An adenoma (upper left corner) was found within a papillary carcinoma (lower right corner).


3.4.48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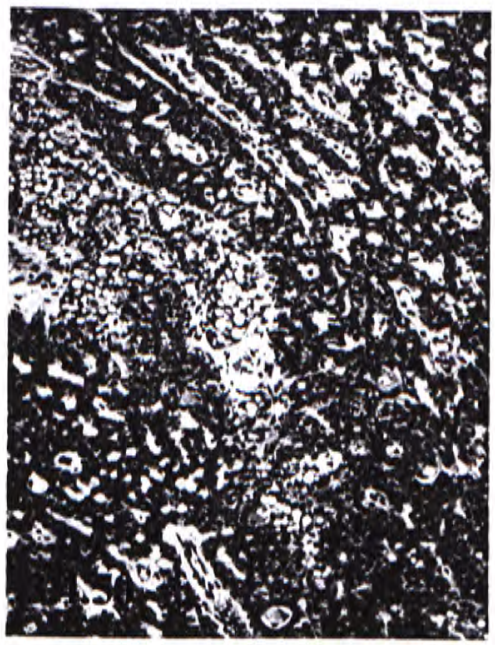


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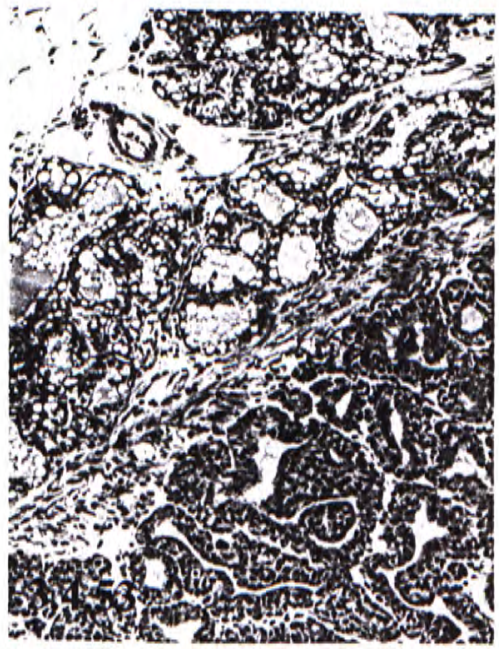
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Figure 3.4.54 & 3.4.55 Early Histological Changes in Hormone-Treated Mammary Glands. There was extensive formation of the enlarged cystic lobules. The histology of Individual alveolus of the lobule was relatively normal. Stroma surrounding the cystic alveoli was composed of normal adipose cells

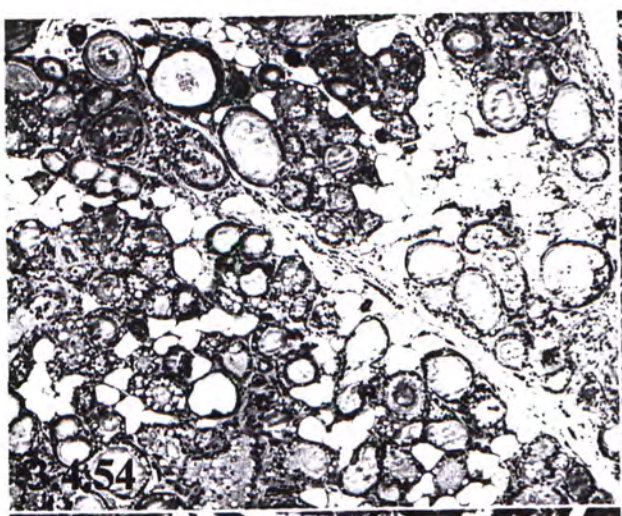
Figure 3.4.56 & 3.4.57 Details of Individual Alveoli in Hormone-Induced Cystic Lobules. The histology of alveoli was relatively normal. The white arrow in figure 3.4.56 indicated that the alveolar epithelium was cuboidal in shape and was one layer thick. The black arrow in figure 3.4.56 indicated an early atypical epithelial hyperplasia observed in adjacent alveolus. Figure 3.4.57 indicated another example of the atypical epithelial hyperplasia. The contour of the alveoli was irregular. The lining epithelium was more than one layer thick.

Figure 3.4.58 Hormone-Induced Mammary Carcinoma, Cribriform Pattern.

Figure 3.4.59 Hormone-Induced Mammary Carcinoma, Mixed Cribriform and Comedo Pattern.

Figure 3.4.60 Hormone-Induced Mammary Carcinoma. Clusters of neoplastic epithelial cells were found in the distended cystic lumen.

Figure 3.4.61 Hormone-Induced Mammary Carcinoma. The core region of the detached carcinoma islets was composed of edematous fibroconnective tissues. Infiltration of lymphocytes was also observed.



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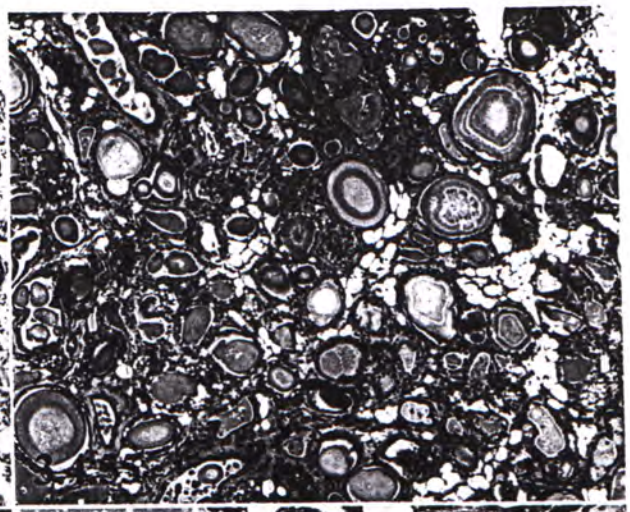

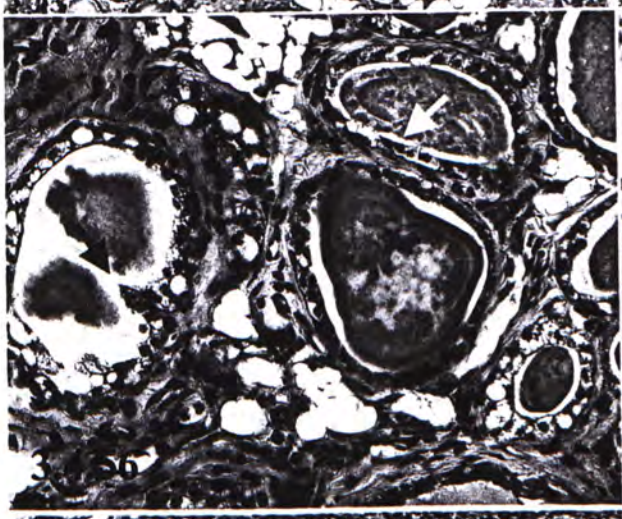

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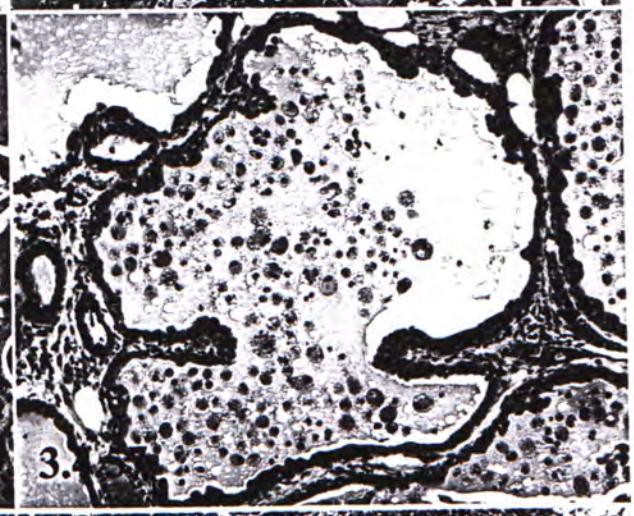

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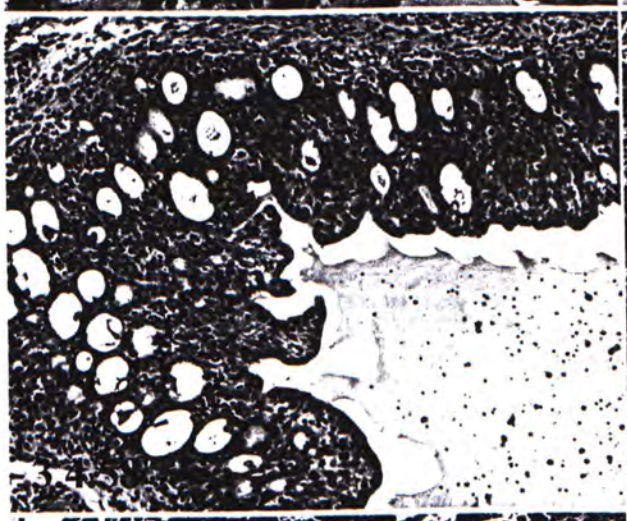

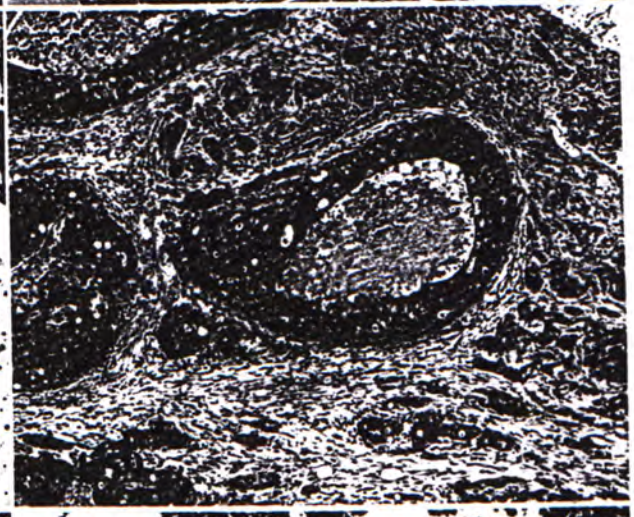

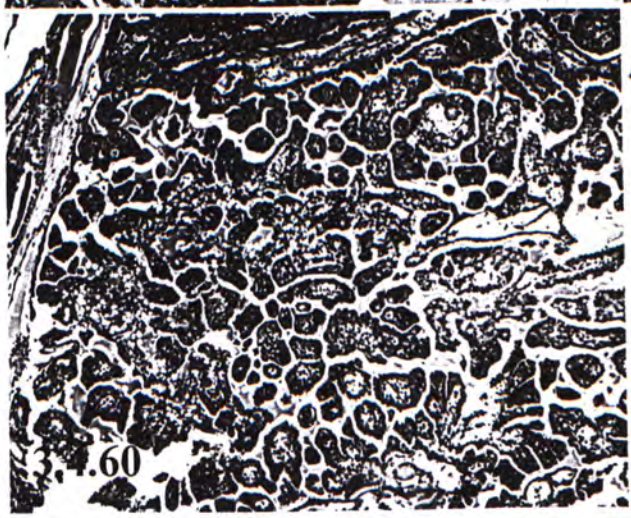

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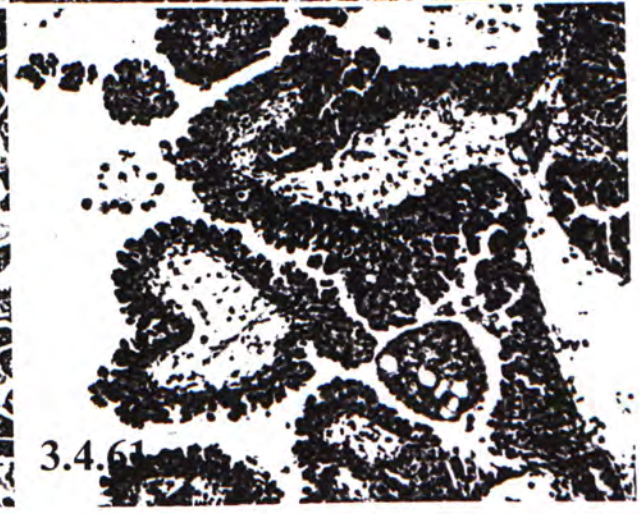

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Figure 3.4.62 – 3.4.65 DMBA-Induced Mammary Carcinoma in Female Noble Rats

Figure 3.4.62 Invasive Ductal Carcinoma, Cribriform Pattern.

Figure 3.4.63 Invasive Ductal Carcinoma, Comedo Pattern.

Figure 3.4.64 Invasive Ductal Carcinoma, Papillary Pattern.

Figure 3.4.65 Anaplastic Carcinoma. Lines of epithelial cells were invading surrounding stromal tissues.

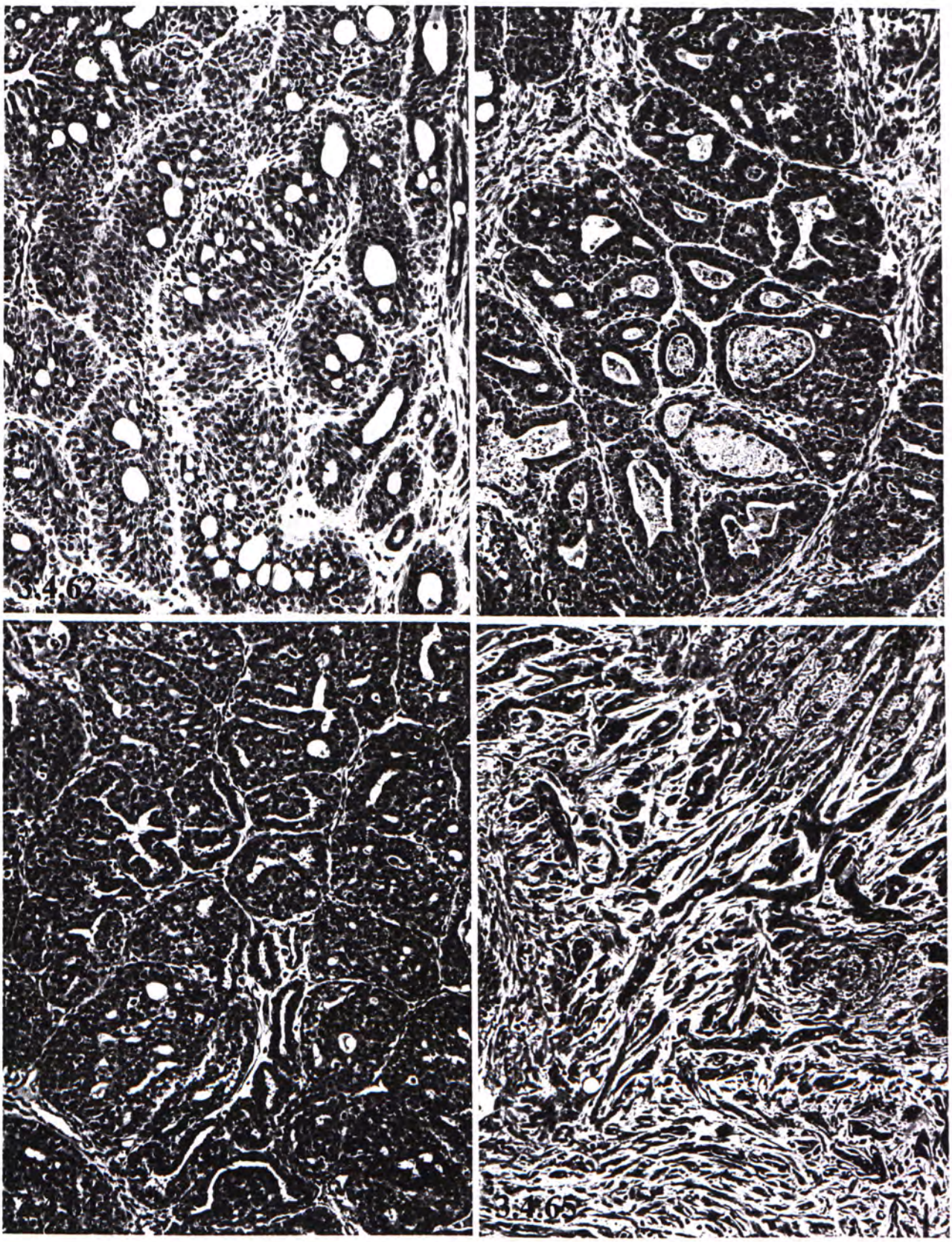
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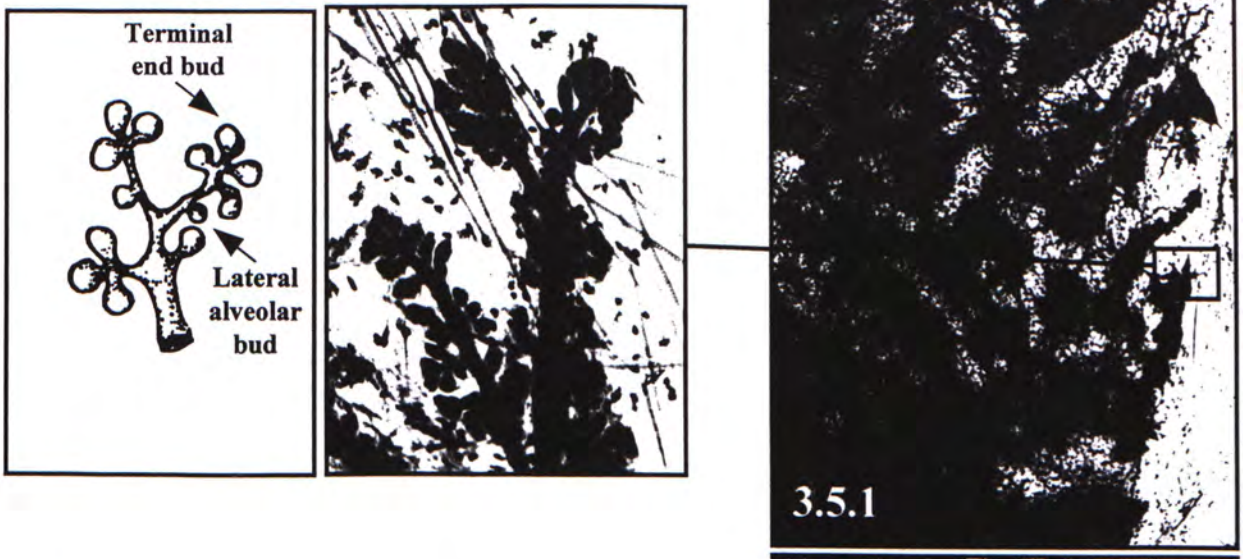
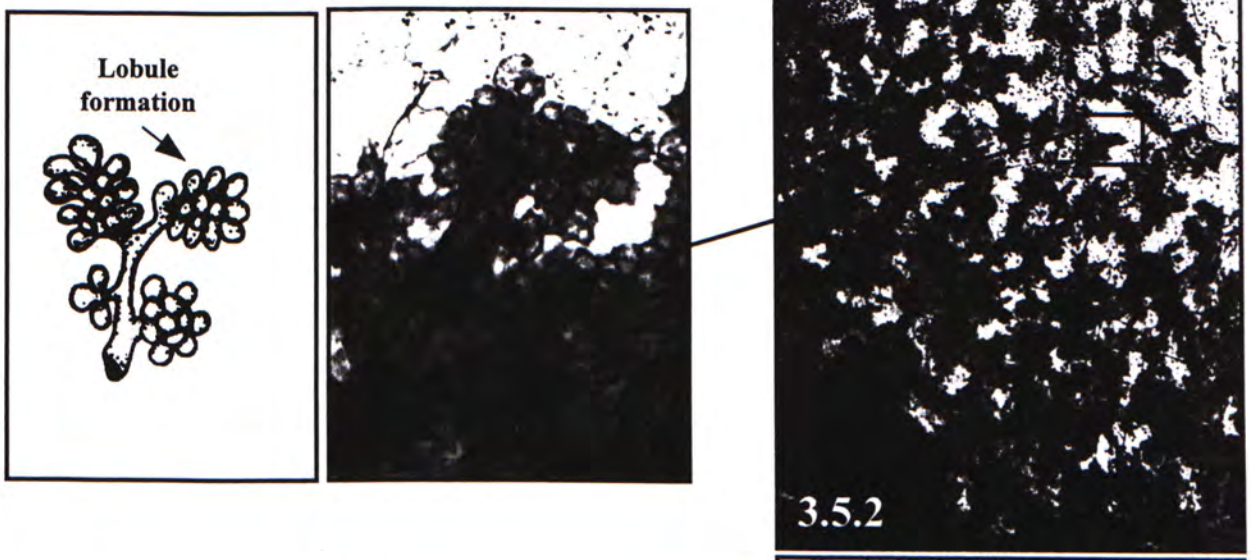
Figure 3.5.1 – 3.5.3 Whole Mount Preparation of Noble Rat Mammary Gland.

Figure 3.5.1 Intact Virgin Noble Rat Mammary Gland. The gland contained numerous sparsely distributed, club-shaped lateral alveolar buds and terminal end buds.

Figure 3.5.2 Virgin Noble Rat Mammary Gland After 10-day T+E_2 Treatment. After short-term hormonal treatment, the cystic alveolar buds increased in number and size. The terminal ducts were also thickened. Terminal buds were not found at the ending of the terminal duct.

Figure 3.5.3 Virgin Noble Rat Mammary Gland After 2-month T+E_2 Treatment. Prolonged hormonal treatment resulted in further increase in the number and size of the cystic alveolar buds.


Terminal
end bud
Lateral
alveolar
bud
3.5.1


Lobule
formation
3.5.2

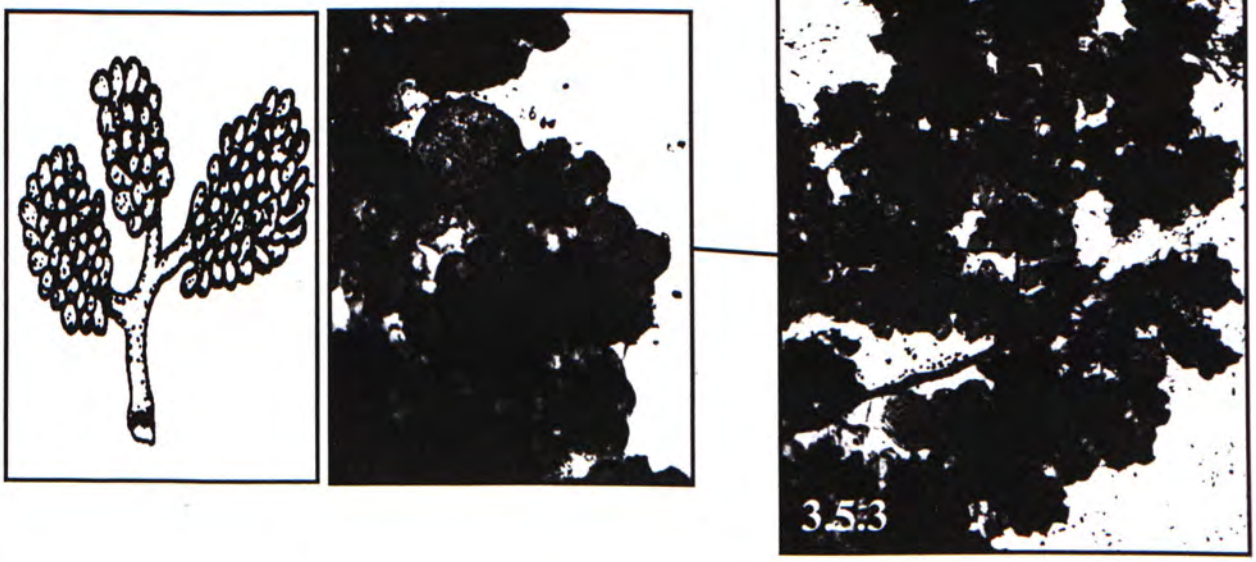

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Figure 3.6 Effect of Bilateral Ovariectomy on the Growth of Spontaneously Developed Mammary Tumors in Female Noble Rats

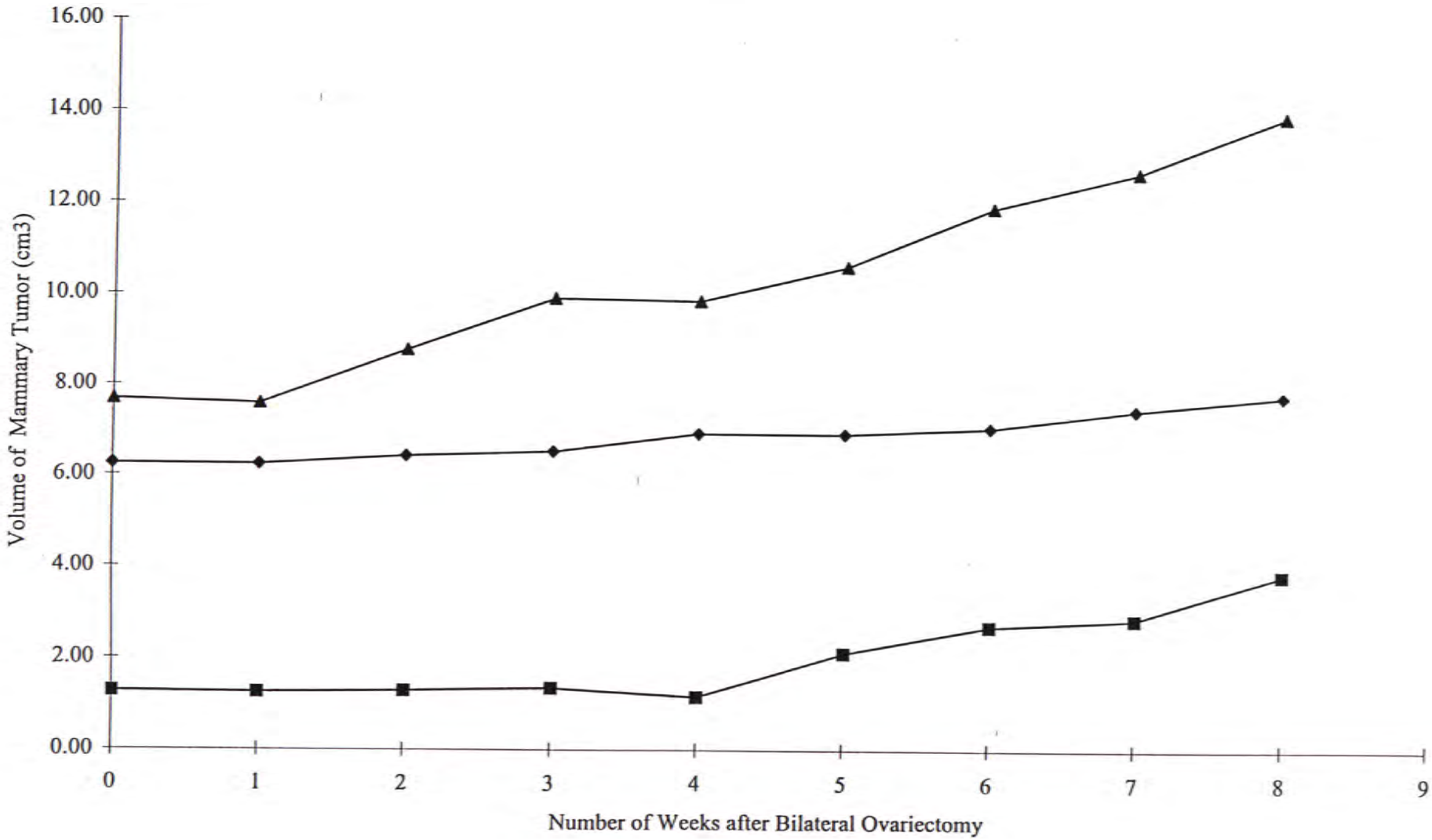
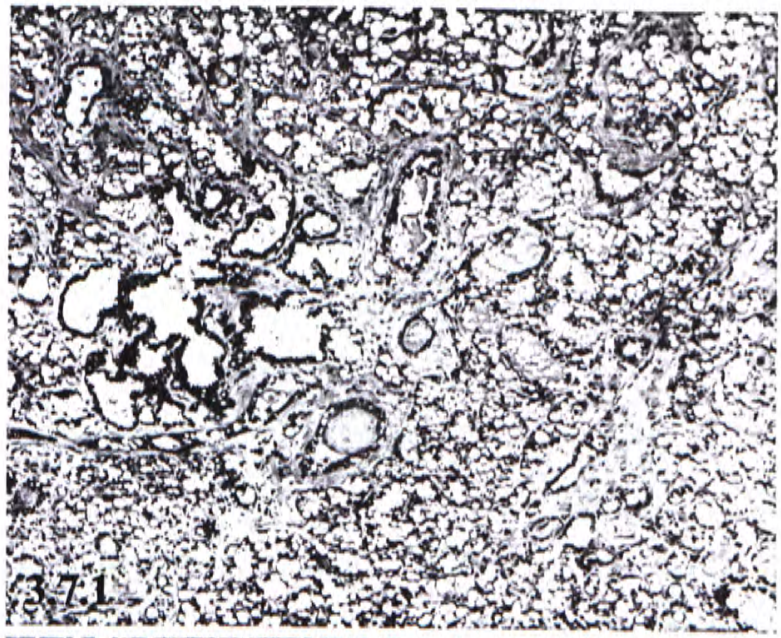


Figure 3.7.1 Spontaneously Developed Benign Secretory Adenoma From a 14 Months Old Female Noble Rat. The alveolar Lumina were dilated and contained plenty of secretory materials. Only scanty connective tissue was present.

Figure 3.7.2 Spontaneously Developed Benign Secretory Fibroadenoma From a 12 Months Old Female Noble Rat. The secretory tubular alveolar components were separated by septa of connective tissue.

Figure 3.7.3 Spontaneously Developed Benign Nonsecretory Fibroadenoma From a 13 Months Old Female Noble Rat. Concentric layers of dense connective tissue compressed the non-secretory tubular alveolar components.



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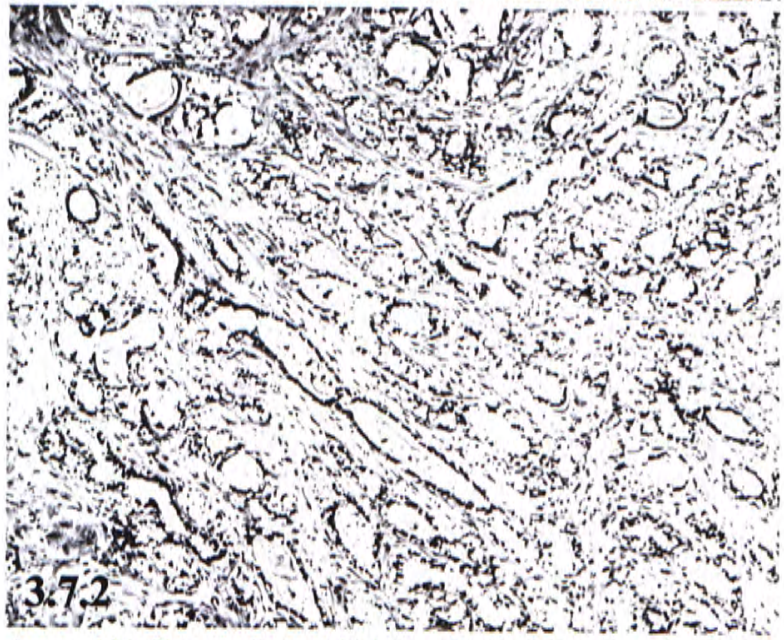

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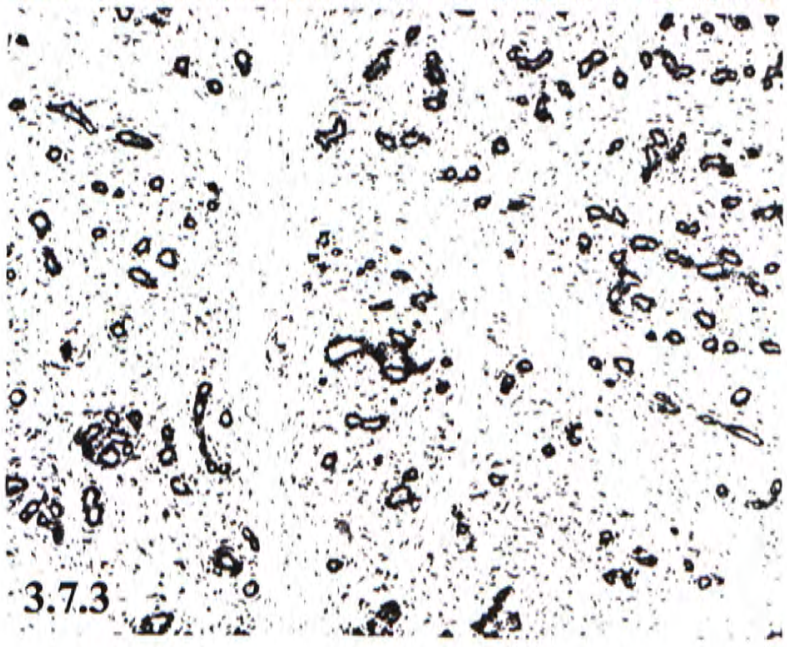

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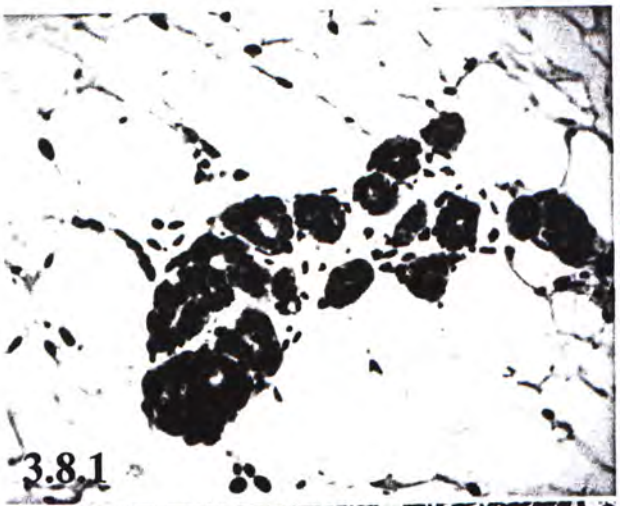

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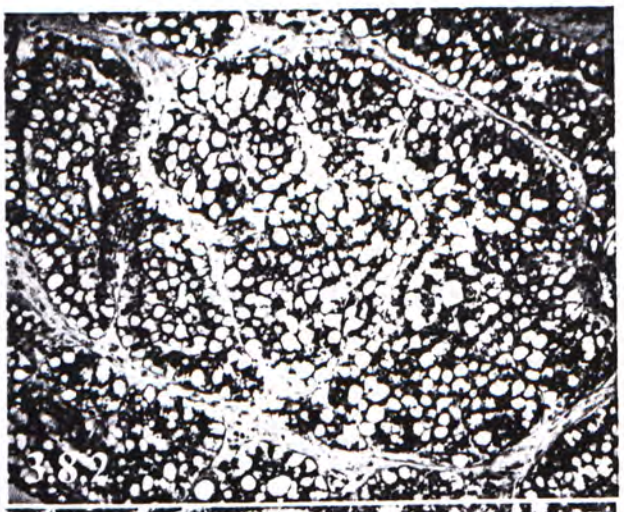

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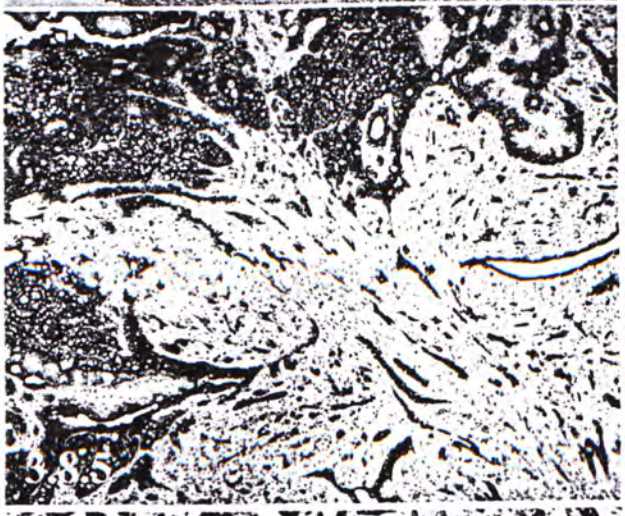

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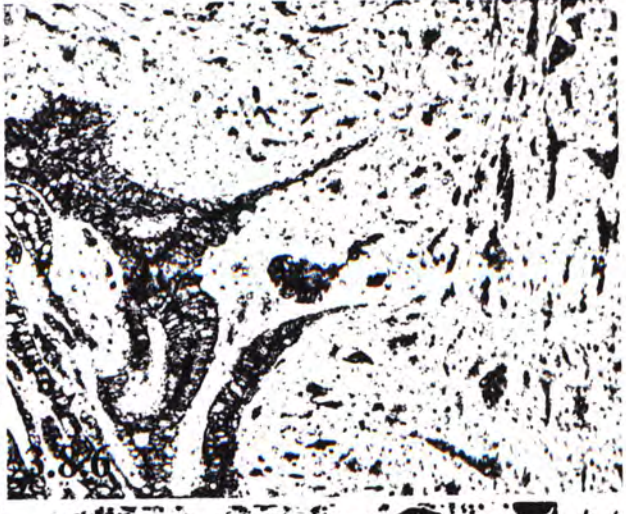

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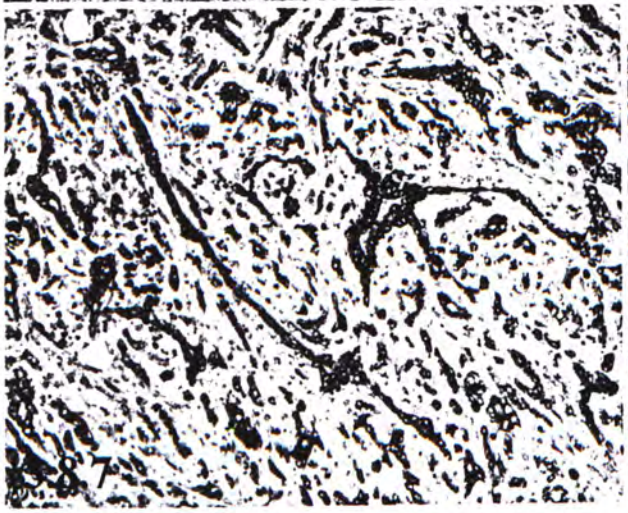

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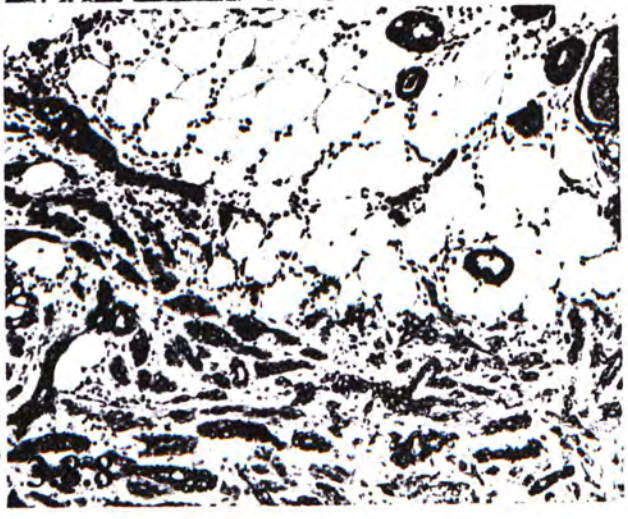

3.8.8

Figure 3.8.1 – 3.8.8 Immunohistochemical Analysis of Epithelial Keratin Expression In Spontaneously Developed Mammary Tumors

Figure 3.8.1 Normal Female Noble Rat Mammary Gland. The cytoplasm of the normal mammary epithelial cells was positively stained for keratins.

Figure 3.8.2 Spontaneously Developed Benign Fibroadenoma. The cytoplasm of the alveolar epithelial cells was strongly positively stained with keratin.

Figure 3.8.3 & 3.8.4 Spontaneously Developed Carcinoma *In Situ.* The papillary tumor cells were well circumscribed by stromal tissues. The cytoplasm of individual epithelial cells was positively stained with keratin.

Figure 3.8.5 & 3.8.6 Invasive Ductal Carcinoma. Clusters of epithelial cells were invading surrounding stromal tissue. The cytoplasm of the carcinoma cells was heavily stained with keratins.

Figure 3.8.7 Anaplasic Carcinoma. As a result of the invasion, islets of positively stained epithelial cells could be found in the stromal cells.

Figure 3.8.8 Invasion of Anaplastic Carcinoma. Individual positively stained anaplastic carcinoma cells were invading surrounding normal mammary stromal tissues.

Figure 3.9.1 – 3.9.2 Negative Control for Immunohistochemsitry. Primary Antibody was omitted to examine the specificity of the immunohistochemical signals. Immunoreactivity was not observed in the negative control samples.

Figure 3.9.1 Female Noble Rat Uterus.

Figure 3.9.2 Female Noble Rat Mammary Gland.

Figure 3.9.3 – 3.9.8 Expression and Localization of Hormone Receptors in Control Tissue.

Figure 3.9.3 & 3.9.4 Immunohistochemistry of Estrogen Receptor α in Adult Noble Rat Uterus. Strong nuclear staining was observed in most endometrial and stromal cells

Figure 3.9.5 & 3.9.6 Immunohistochemistry of Estrogen Receptor β in Adult Noble Rat Uterus. Strong nuclear staining was observed in most endometrial cells, stromal cells and skeletal muscle cells.

Figure 3.9.7 & 3.9.8 Immunohistochemistry of Progesterone Receptor in Adult Noble Rat Uterus. The stromal and muscle uterine cells were positively stained.

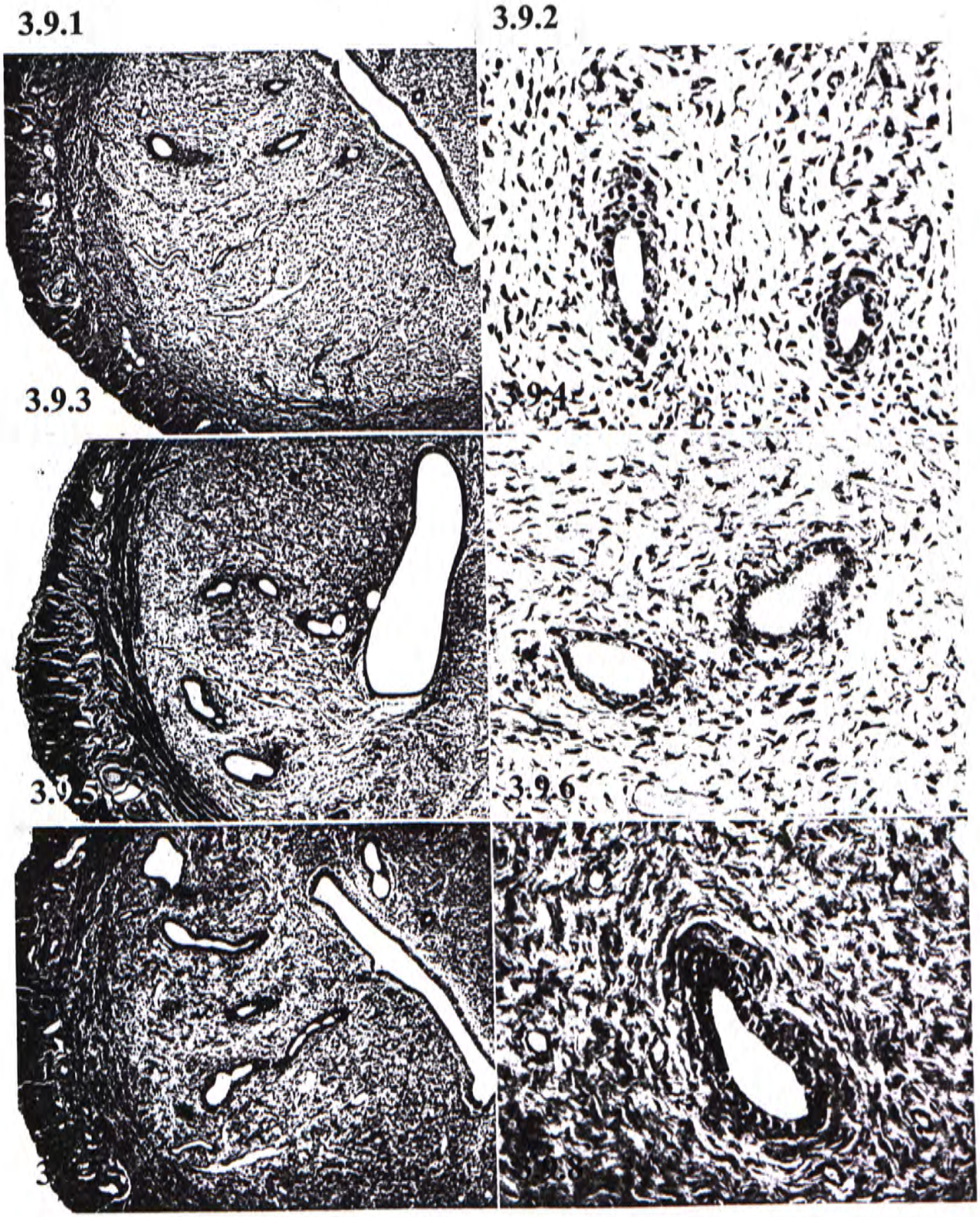
3.9.1
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Figure 3.9.9 – 3.9.14 Immunohistochemical Analysis of Expression and Localization of Estrogen Receptor α in Mammary Glands and Tumors of Noble Rats.

Figure 3.9.9 Normal Female Noble Mammary Gland. Intense nuclear immunostaining was observed in the normal mammary epithelial cells.

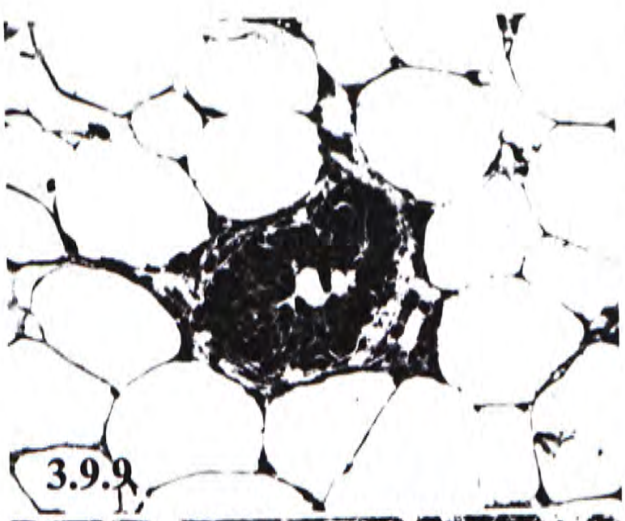
Figure 3.9.10 Spontaneously Developed Benign Tumor. Strong nuclear immunostaining was observed in the alveolar epithelial tumor cells.

Figure 3.9.11 Spontaneously Developed Malignant Carcinoma. The nuclei of carcinoma cells were heavily positively stained.

Figure 3.9.12 DMBA-Induced Malignant Carcinoma. Moderate immunoreactivity was observed in both carcinoma and stromal cells.

Figure 3.9.13 T+E_2-Induced Malignant Carcinoma. The nuclei of the carcinoma cells were strongly stained.

Figure 3.9.14 T+DES-Induced Malignant Carcinoma. The cytoplasm and nuclei of the carcinoma cells were strongly stained.



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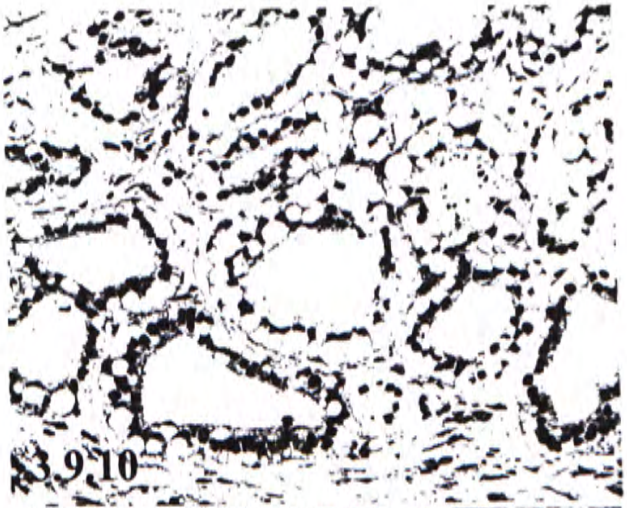

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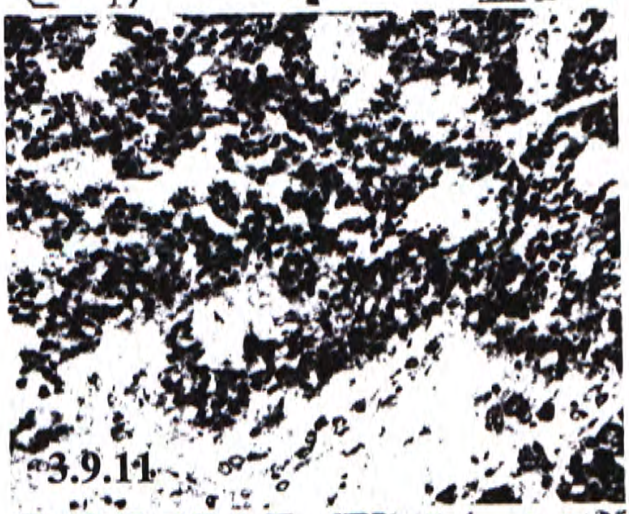

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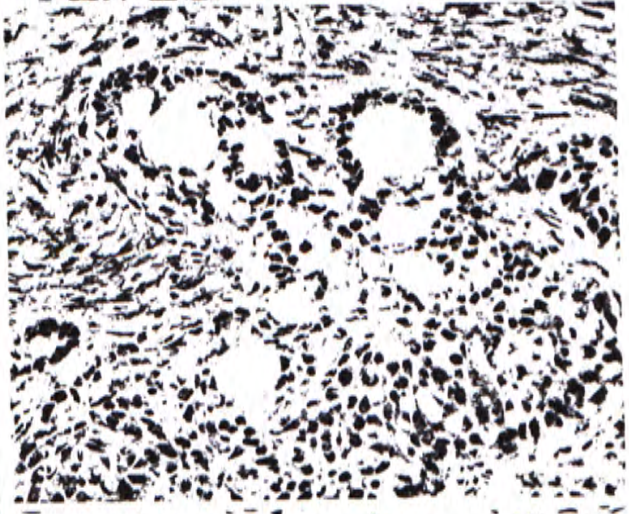

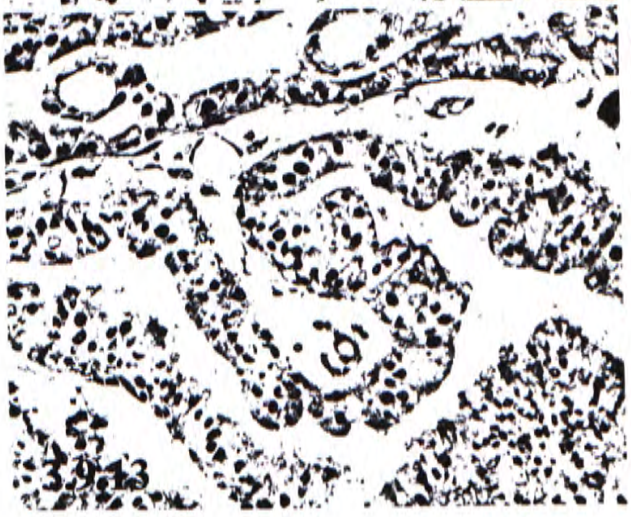

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Figure 3.9.15 – 3.9.20 Immunohistochemical Analysis of Expression and Localization of Estrogen Receptor β in Mammary Glands and Tumors of Noble Rats.

Figure 3.9.15 Normal Female Noble Mammary Gland. Moderate cytoplasmic and nuclear staining was observed in the ductal epithelial cells.

Figure 3.9.16 Spontaneously Developed Benign Tumor. The cytoplasm of the benign tumor cells only exhibited weak to moderate staining.

Figure 3.9.17 Spontaneously Developed Malignant Carcinoma. Moderate cytoplasmic staining was observed in the ductal carcinoma cells.

Figure 3.9.18 DMBA-Induced Malignant Carcinoma. Only very low immunoreactivity was observed in the carcinoma cells

Figure 3.9.19 T + E_2-Induced Malignant Carcinoma. The carcinoma cells exhibited very strong cytoplasmic staining.

Figure 3.9.20 T+DES-Induced Malignant Carcinoma. Intense cytoplasmic immunoreactivity was observed in the carcinoma cells.

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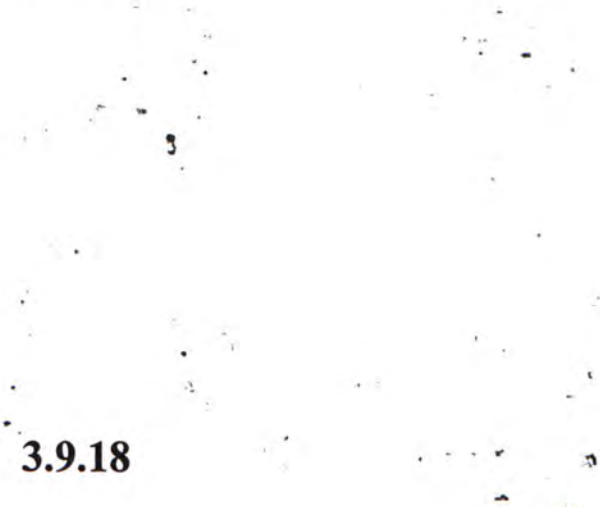

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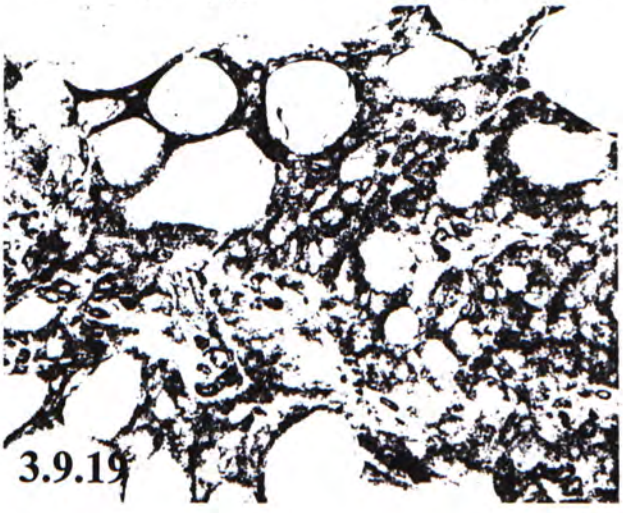

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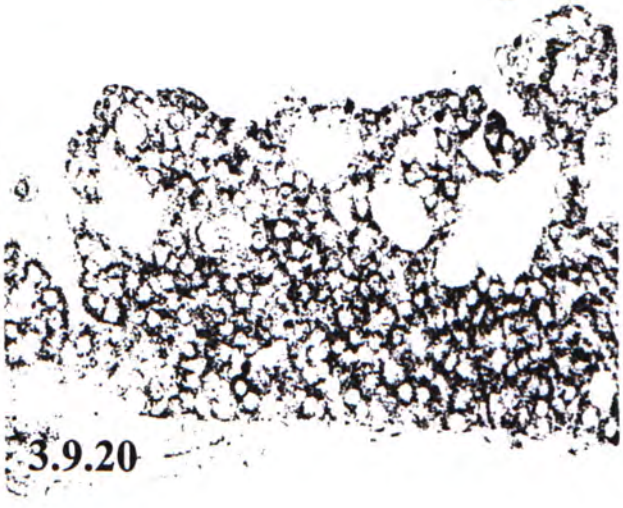

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Figure 3.9.21 – 3.9.26 Immunohistochemical Analysis of Expression and Localization of Progesterone Receptor in Mammary Glands and Tumors of Noble Rats.

Figure 3.9.21 Normal Female Noble Mammary Gland. Moderate nuclear immunostaining was observed in the normal mammary epithelial cells.

Figure 3.9.22 Spontaneously Developed Benign Tumor. Strong nuclear immunostaining was observed in the alveolar epithelial tumor cells.

Figure 3.9.23 Spontaneously Developed Malignant Carcinoma. The nuclei of carcinoma cells were heavily positively stained.

Figure 3.9.24 DMBA-Induced Malignant Carcinoma. Moderate immunoreactivity was observed in carcinoma cells.

Figure 3.9.25 T+E_2-Induced Malignant Carcinoma. The nuclei of the carcinoma cells were moderately stained.

Figure 3.9.26 T+DES-Induced Malignant Carcinoma. The nuclei of the carcinoma cells were strongly stained.

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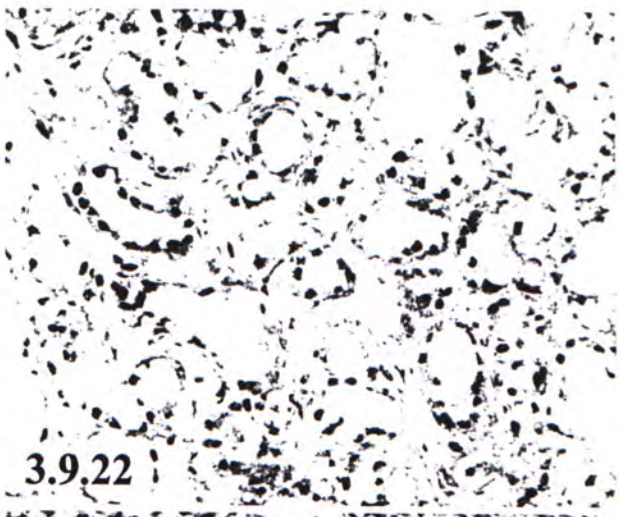

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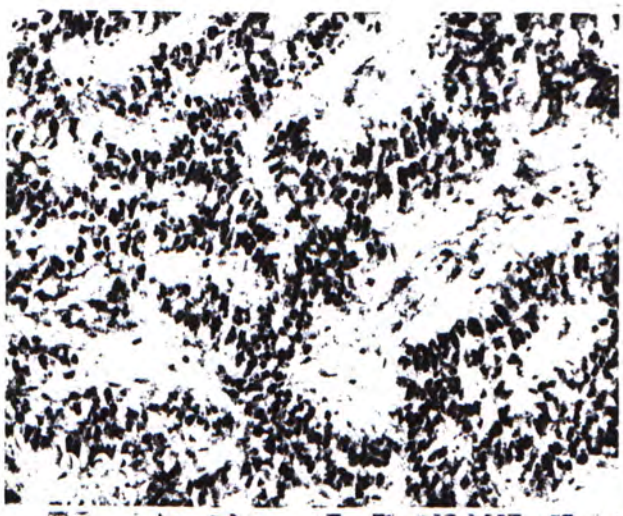

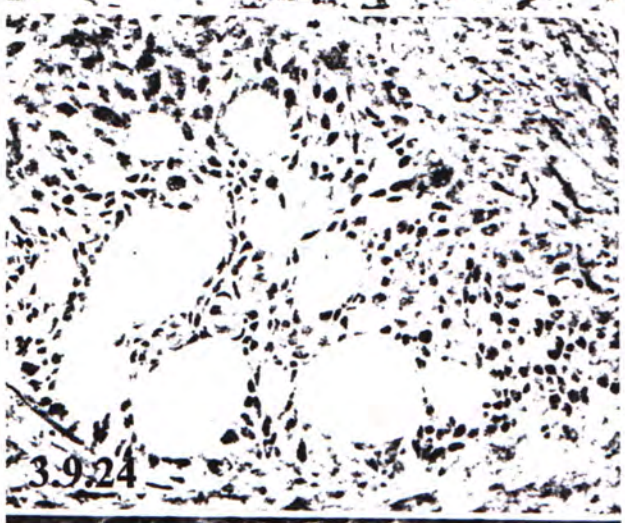

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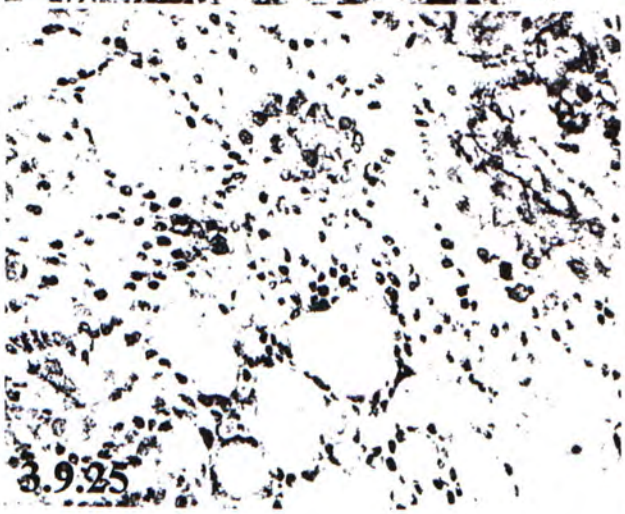

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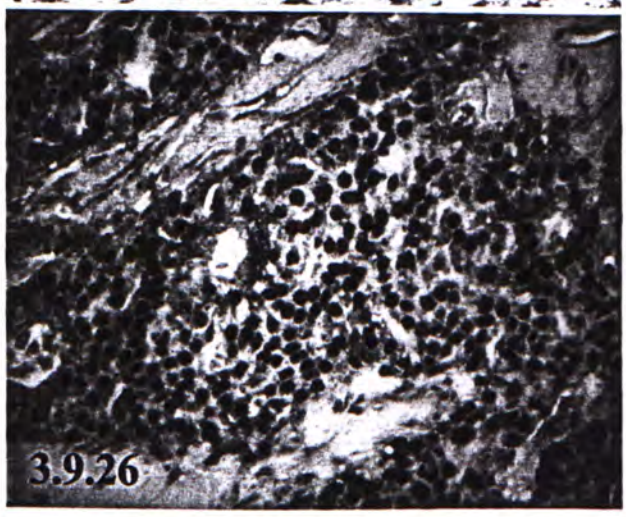

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Figure 3.9.27 Negative Control for Immunohistochemsitry of Androgen Receptor. Primary Antibody was omitted to examine the specificity of the immunohistochemical signals on rat prostate. Immunoreactivity was not observed in the negative control sample.

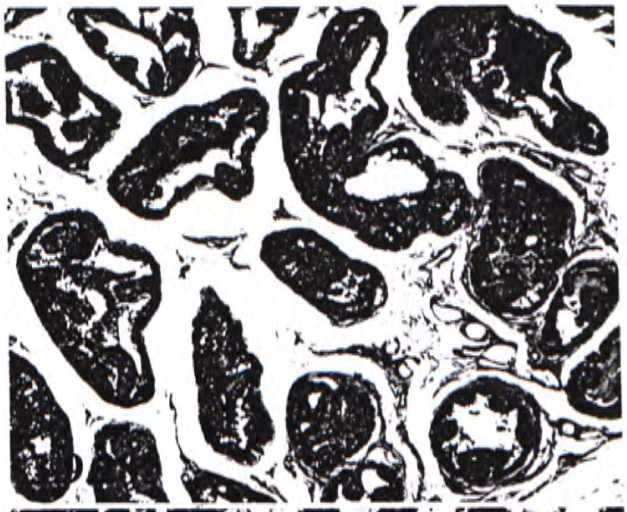
Figure 3.9.28 Immunohistochemistry of Androgen Receptor in Adult Noble Rat Prostate. Strong nuclear and cytoplasmic immunoreactivity were observed in the glandular epithelium.

Figure 3.9.29 Negative Control for Immunohistochemsitry of Prolactin Receptor in Lactating Mammary Glands. Immunoreactivity was not detected in the negative control sample.

Figure 3.9.30 Immunohistochemistry of Prolactin Receptor in Lactating Noble Rat Mammary Gland. Strong immunoreactivity was observed in the cell membranes of the alveolar epitheliums.

Figure 3.9.31 Negative Control for Immunohistochemsitry of Prolactin Receptor in Rat Liver. Immunoreactivity was not detected in the negative control sample.

Figure 3.9.32 Immunohistochemistry of Prolactin Receptor in Female Noble Rat Liver. Strong immunoreactivity was observed in the cell membranes and cytoplasmic granules of the hepatocytes.



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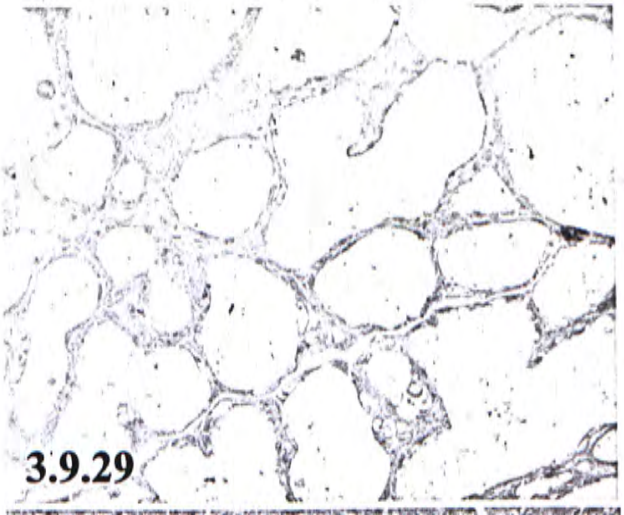

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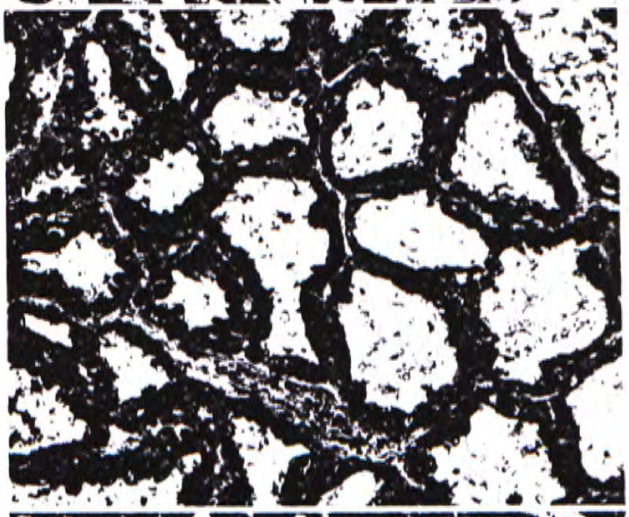

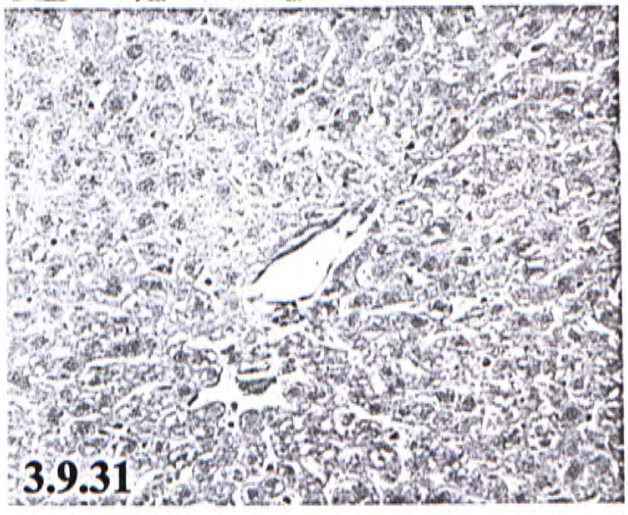

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Figure 3.9.33 – 3.9.34 Immunohistochemical Analysis of Expression and Localization of Androgen Receptor in Mammary Glands and Tumors of Noble Rats.

Figure 3.9.33 Normal Female Noble Mammary Gland. Only moderate immunoreactivity was observed in the epithelial cells.

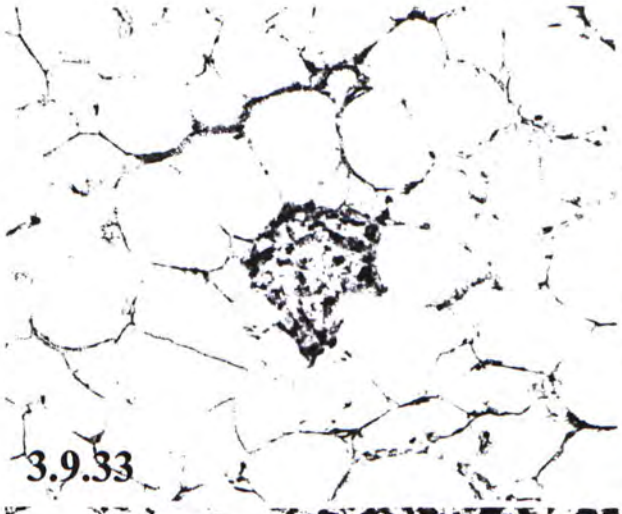
Figure 3.9.34 Spontaneously Developed Benign Tumor. Strong nuclear and cytoplasmic immunoreactivity was observed in the alveolar epithelial tumor cells.

Figure 3.9.35 Spontaneously Developed Malignant Carcinoma. The nuclei of the carcinoma cells were heavily stained.

Figure 3.9.36 DMBA-Induced Malignant Carcinoma. Moderate immunoreactivity was observed in the carcinoma cells.

Figure 3.9.37 T+E_2-Induced Malignant Carcinoma. The nuclei of the carcinoma cells were moderately stained for AR.

Figure 3.9.38 T+DES-Induced Malignant Carcinoma. The nuclei and cytoplasm of the carcinoma cells were strongly stained for AR



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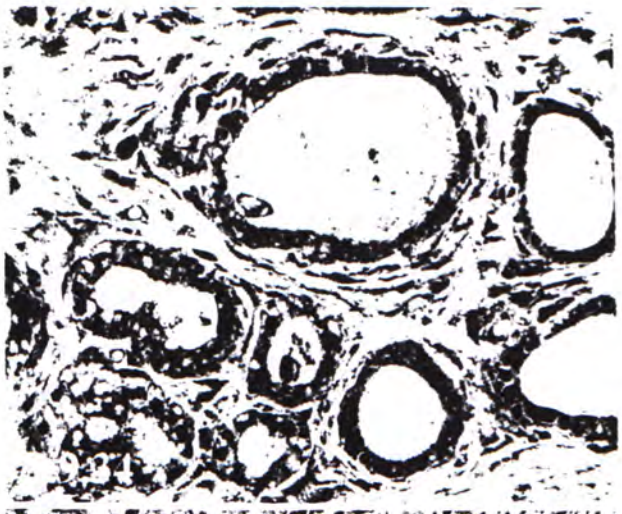

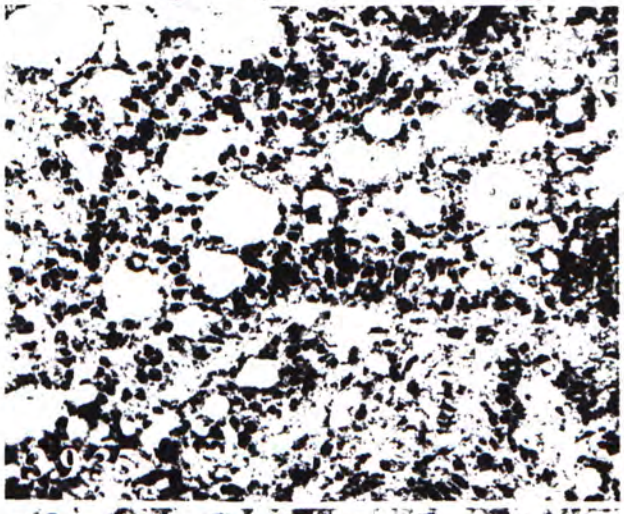

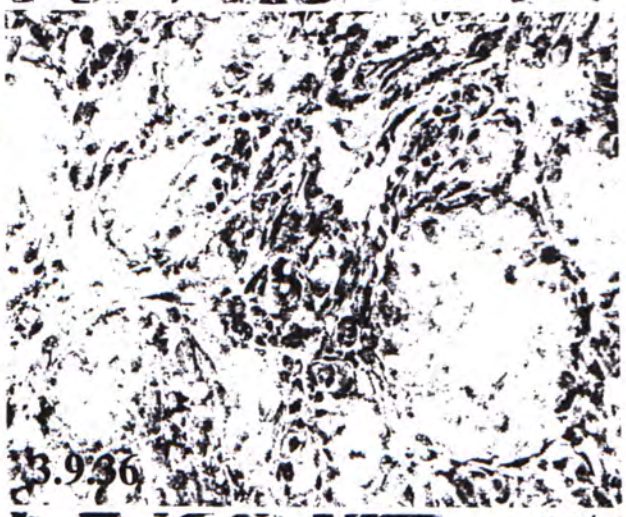

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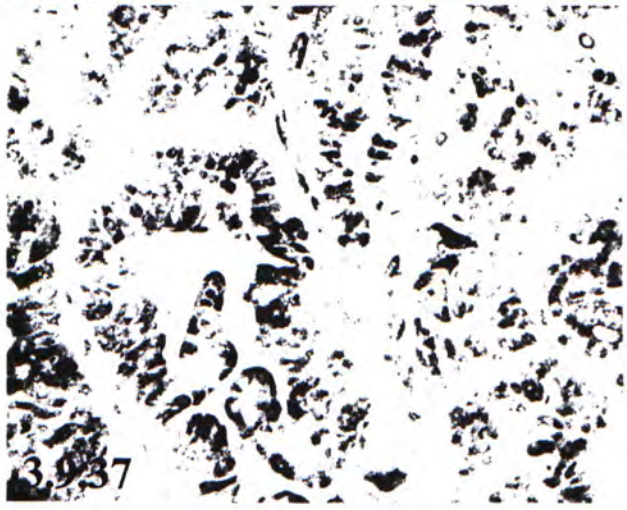

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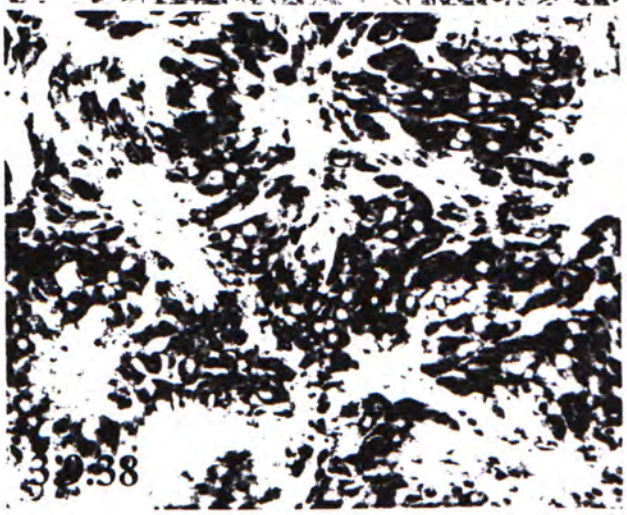

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Figure 3.9.39 – 3.9.44 Immunohistochemical Analysis of Expression and Localization of Prolactin Receptor in Mammary Glands and Tumors of Noble Rats.

Figure 3.9.39 Normal Female Noble Mammary Gland. Weak to moderate cytoplamic immunoreactivity was observed in the normal mammary epithelial cells.

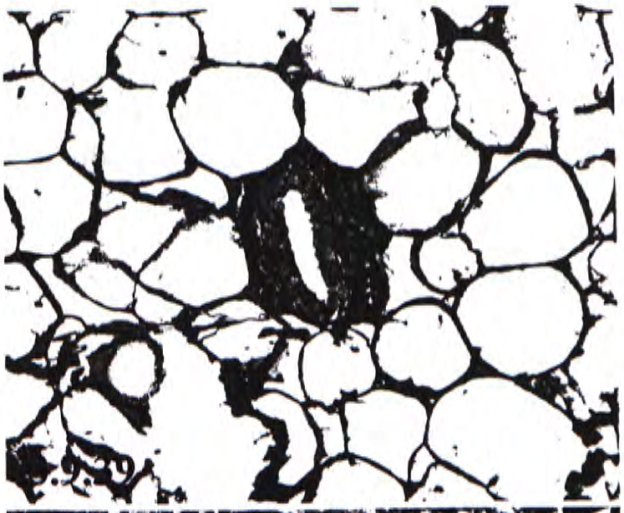
Figure 3.9.40 Spontaneously Developed Benign Tumor. Strong immunoreactivity was observed in the plasma membranes and cytoplasm of most epithelial tumor cells.

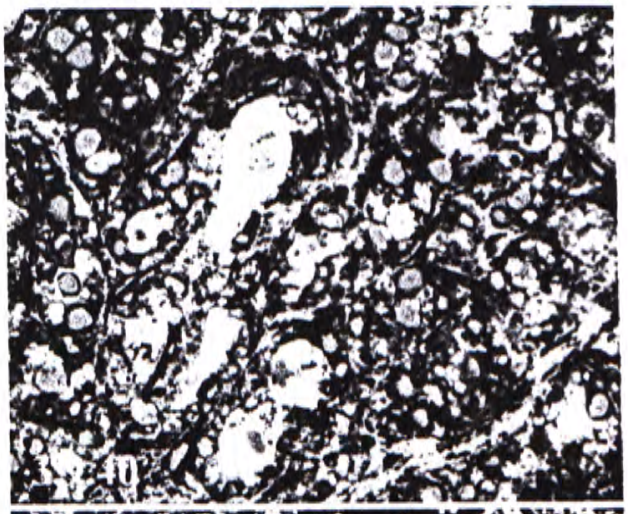
Figure 3.9.41 Spontaneously Developed Malignant Carcinoma. The plasma membrane and cytoplasm of carcinoma cells were heavily stained for PRLR.

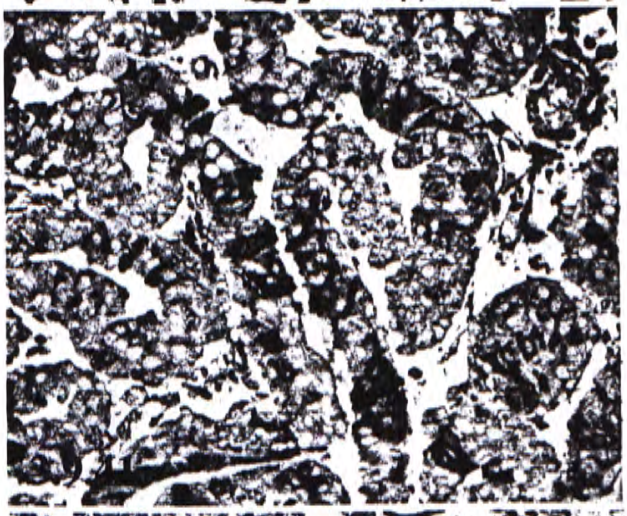
Figure 3.9.42 DMBA-Induced Malignant Carcinoma. Strong PRLR immunoreactivity was observed in focal areas.

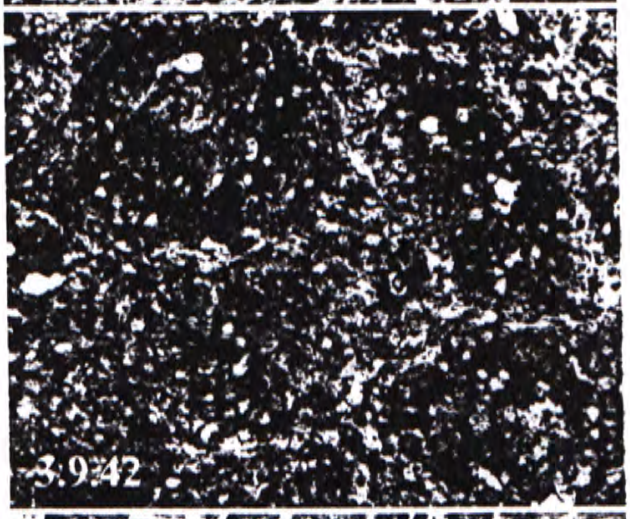
Figure 3.9.43 T+E_2-Induced Malignant Carcinoma. The cytoplasm of the carcinoma cells was weakly to moderately stained for PRLR.

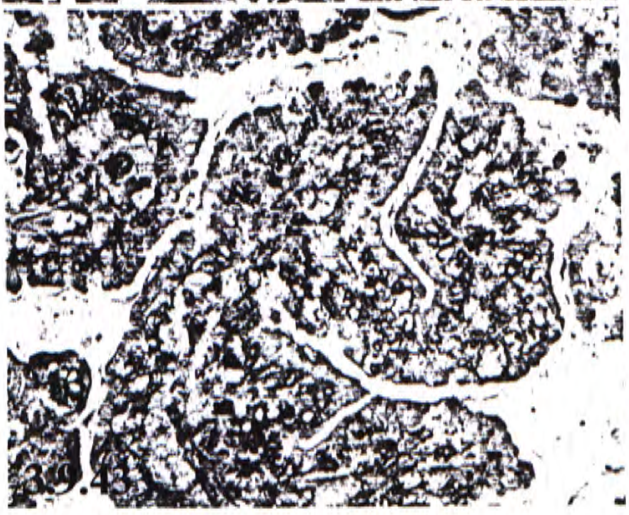
Figure 3.9.44 T+DES-Induced Malignant Carcinoma. The cytoplasm and plasma membrane of the carcinoma cells were strongly stained.











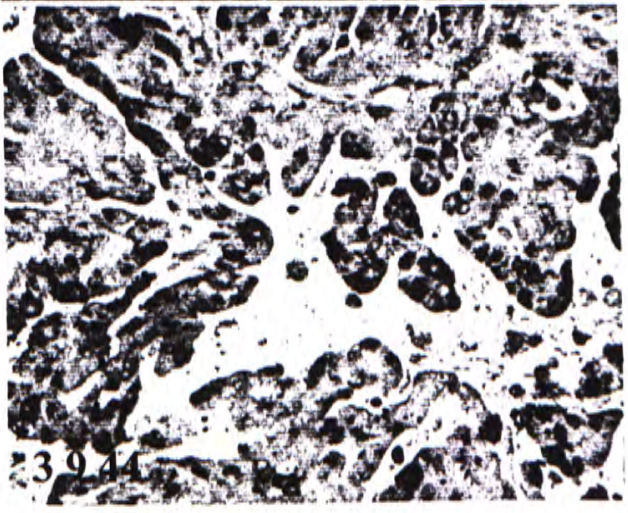


Figure 3.10.1 Western Blot Analysis of Expression of Estrogen Receptor α Proteins in Normal and Neoplastic Mammary Tissues of Female Noble Rats

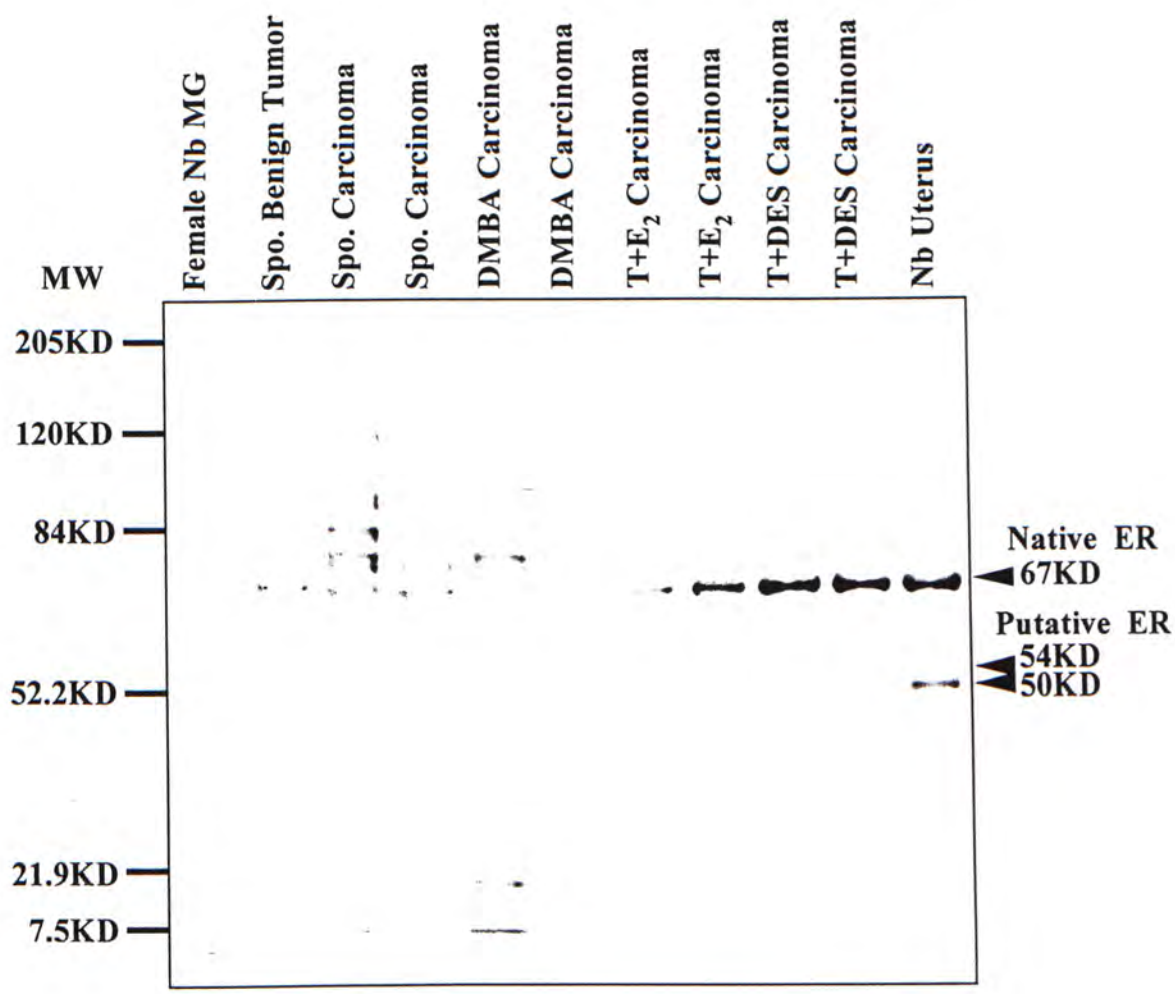


Figure 3.10.2 Western Blot Analysis of Expression of Estrogen Receptor β Proteins in Normal and Neoplastic Mammary Tissues of Female Noble Rats

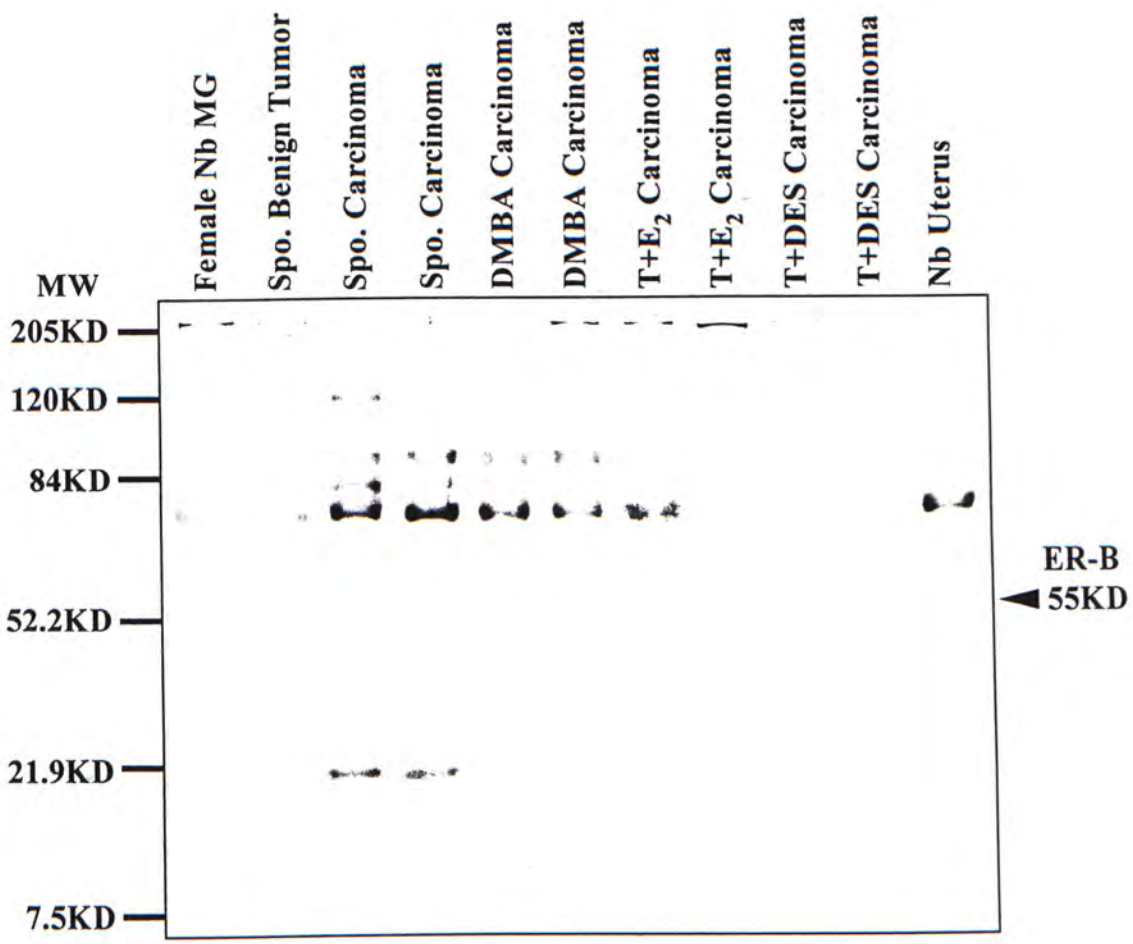


Figure 3.10.3 Western Blot Analysis of Expression of Progesterone Receptor Proteins in Normal and Neoplastic Mammary Tissues of Female Noble Rats

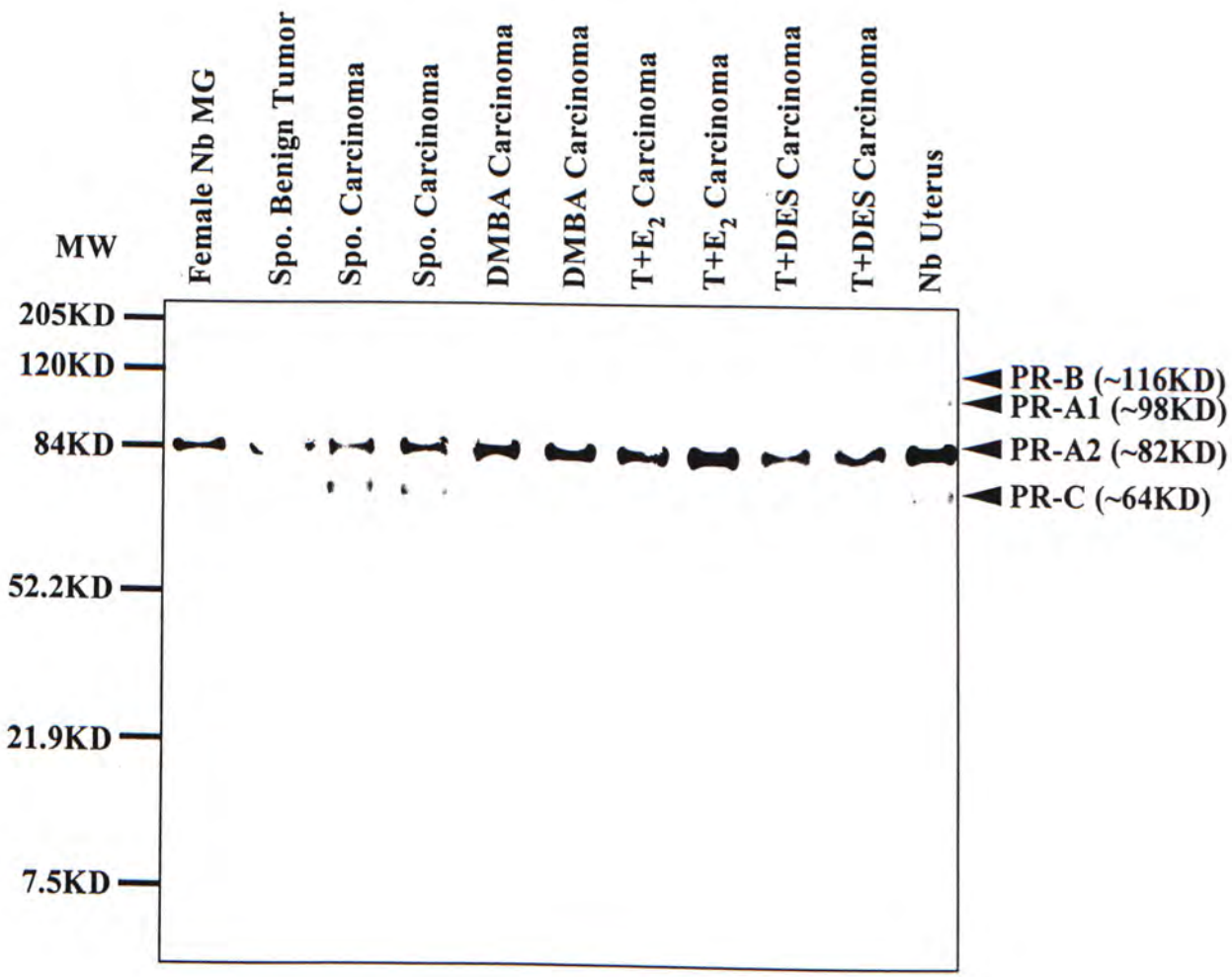


Figure 3.10.4 Western Blot Analysis of Expression of Androgen Receptor Proteins in Normal and Neoplastic Mammary Tissues of Female Noble Rats

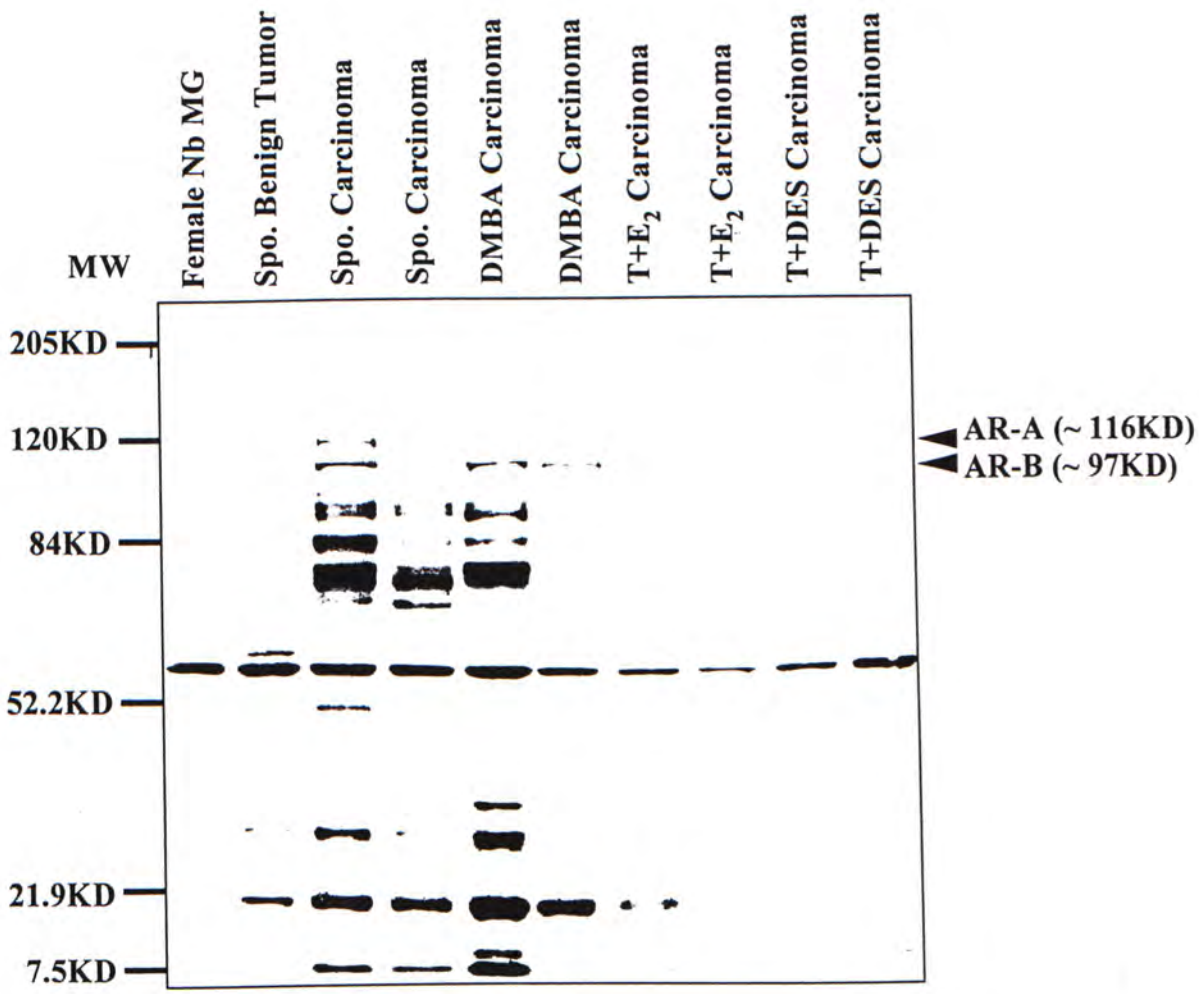


Figure 3.10.5 Western Blot Analysis of Expression of Prolactin Receptor Proteins in Normal and Neoplastic Mammary Tissues of Female Noble Rats

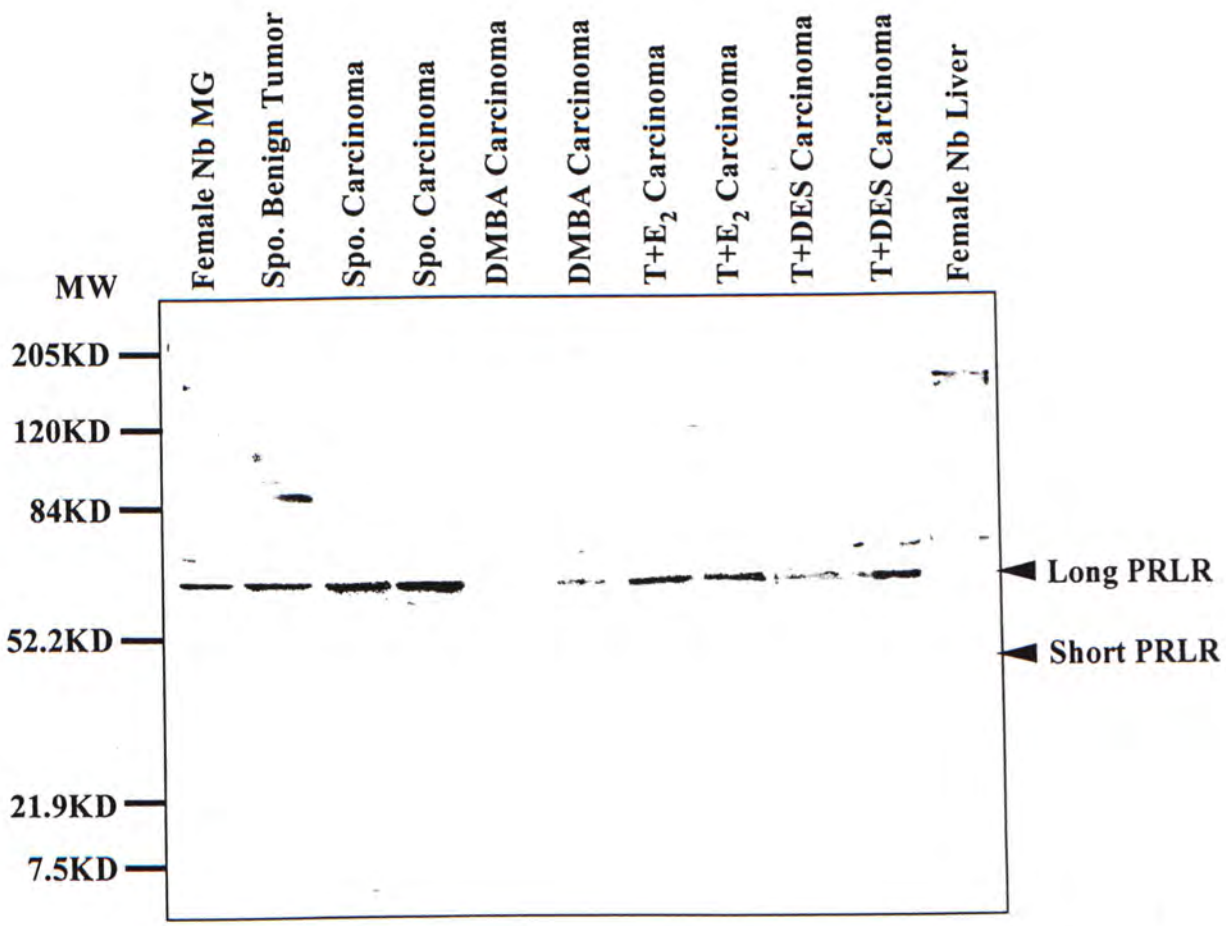


Table 3.1. Incidence rate, Distribution and Histopathology of the Spontaneously Developed Mammary Tumors in Female and Male Noble Rats

Nb rat	Age	Mammary tumor location	Histopathology
1. Female	8 months old	Left thoracic gland	Benign Fibroma
2. Female	12 months old	Left abdominal gland	Benign fibroadenoma
3. Female	12 months old	Right thoracic gland Left thoracic gland	Benign Fibroadenoma Benign Fibroadenoma
4. Female	13 months old	Right abdominal gland	Malignant invasive ductal carcinoma
5. Female	13 months old	Right upper thoracic mammary gland Right lower thoracic mammary gland	Benign Fibroadenoma Benign Fibroadenoma
6. Female	14 months old	Right thoracic mammary gland	Benign Fibroadenoma
7. Female	14 months old	Right thoracic mammary gland Left thoracic mammary gland	Benign adenoma Benign Fibroadenoma
8. Female	14 months old	Left thoracic mammary gland	Benign Fibroadenoma
9. Female	14 months old	Left thoracic mammary gland	Benign Fibroadenoma
10. Female	14 months old	Right inguinal mammary gland	Malignant papillary adenocarcinoma
11. Female	14.5 months old	Left abdominal mammary gland	Malignant papillary Adenocarcinoma
12. Female	15 months old	Right upper thoracic mammary gland Right lower thoracic mammary gland Right abdominal mammary gland Right inguinal mammary gland	Benign Fibroadenoma Benign Fibroadenoma Benign Fibroadenoma Benign Fibroadenoma
13. Female	15 months old	Right thoracic mammary gland	Benign Fibroma
14. Female	15 months old	Left thoracic mammary gland	Benign Fibroadenoma
15. Female	15 months old	Left abdominal mammary gland	Benign Fibroadenoma
16. Female	16 months old	Right thoracic mammary gland Left thoracic mammary gland Right inguinal mammary gland	Benign Fibroadenoma Benign Fibroadenoma Benign Fibroadenoma
17. Female	18 months old	Left thoracic mammary gland	Malignant papillary adenocarcinoma
18. Female	18 months old	Left inguinal mammary gland	Malignant tubular adenocarcinoma
19. Male	12 months old	Right abdominal mammary gland	Benign Fibroadenoma

Table 3.2. Incidence Rate, Latency Period and Distribution of Mammary Tumors in Female Noble Rats Under Combined Treatment of Testosterone and 17β-Estradiol

Treatment $T+E_2$	Age	Latency Period	Mammary lesion locations	Other Observations
1.	11 months old	8 months	Right upper thoracic gland: fluid lesion Right lower thoracic gland: solid tumor Right abdominal gland: fluid lesion Left thoracic gland: fluid lesion Left abdominal gland: fluid lesion	i. Ovary and uterus appeared abnormal
2.	11 months old	8 months	Right abdominal gland: fluid lesion Left thoracic gland: fluid lesion Left abdominal gland: fluid lesion	i. The rat was moribund
3.	11 months old	8 months	Right thoracic gland: fluid lesion Right abdominal gland: fluid lesion Left thoracic gland: fluid lesion Left abdominal gland: fluid lesion	i. Uterine tumor was found
4.	11 months old	8 months	Left upper thoracic gland: fluid lesion Left lower thoracic gland: fluid lesion Left abdominal gland: fluid lesion	Nil
5.	13 months old	9 months	Right thoracic gland: fluid lesion Right upper abdominal gland: fluid lesion Right lower abdominal gland: solid tumor Left thoracic gland: fluid lesion Left upper abdominal gland: fluid lesion Left lower abdominal gland: solid tumor	Nil
6.	13 months old	10 months	Right cervical gland: fluid lesion Right thoracic gland: fluid lesion Right abdominal gland: fluid lesion Left thoracic gland: fluid lesion Left abdominal gland: fluid lesion	Nil
7.	15 months old	11 months	Right thoracic gland: fluid lesion Right inguinal gland: fluid lesion	i. The rat was moribund ii. Uterus and ovary appeared abnormal iii. Lung also appeared abnormal

Table 3.3. Latency Period and Distribution of Mammary Tumors in Female Noble Rats under Combined treatment of Testosterone and Diethylstilbestrol

Treatment T+DES	Age	Latency Period	Mammary lesion locations	Other Observations
1.	12 months old	9 months	Right upper thoracic gland: fluid lesion Right abdominal gland: fluid lesion Left thoracic gland: solid tumor Left abdominal gland: fluid lesion	i. The rat was moribund ii. Uterus appeared abnormal iii. Lung also appeared abnormal
2.	13 months old	10 months	Right abdominal gland: solid tumor Left cervical gland: solid tumor Left thoracic gland: fluid lesion Left abdominal gland: fluid lesion	i. The rat was moribund ii. Uterus and ovary appeared abnormal iii. Lung and liver also appeared abnormal
3.	13 months old	10 months	Left thoracic gland: solid tumor	i. Uterus and ovary appeared abnormal
4.	13 months old	10 months	Right cervical gland: solid tumor Right thoracic gland: solid tumor Left abdominal gland: fluid lesion	i. The rat was moribund ii. Uterus and ovary appeared abnormal iii. Lung and liver also appeared abnormal
5.	14 months old	11 months	Right abdominal gland: fluid lesion Left thoracic gland: fluid lesion Left abdominal gland: solid tumor	i. Uterus and ovary appeared abnormal

Table 3.4. Incidence Rate And Latency Period of the Hormone Induced Mammary Tumors in Female Noble Rats

Treatment	Number of Female Noble Rats under Hormonal Treatment	Mortality		Incidence Rate		Average Latency Period
$T+E_2$	20	5/20	25%	7/15	46.66%	8.86 months
T+DES	20	11/20	55%	5/9	55.55%	10 months

Table 3.5. Changes in Volume of the Spontaneously Developed Mammary Tumors in the Bilateral Ovariectomized Noble Rats

Weeks after Operation	Bilateral Ovariectomized Noble Rat											
	1				2				3			
	Length (mm)	Width (mm)	Height (mm)	Tumor Volume (cm^3)	Length (mm)	Width (mm)	Height (mm)	Tumor Volume (mm^3)	Length (mm)	Width (mm)	Height (mm)	Tumor Volume (mm^3)
0	28.12	25.81	17.22	6.25	20.33	24.59	5.09	1.27	29.79	28.62	18	7.67
1	28.12	25.81	17.19	6.24	20.35	24.53	5.04	1.26	29.64	28.59	17.91	7.59
2	28.65	26.12	17.16	6.42	19.18	24.76	5.42	1.29	31.72	32.38	17.1	8.78
3	28.45	27.13	16.93	6.53	19.71	25.67	5.31	1.34	33.86	31.04	18.9	9.93
4	29.73	26.34	17.76	6.95	18.89	25.78	4.75	1.16	34.85	30.05	18.9	9.90
5	29.1	25.63	18.65	6.95	19.87	29.24	7.28	2.11	34.99	31.72	19.23	10.67
6	29.21	26.88	18.11	7.11	20.27	30.99	8.59	2.70	35.73	32.64	20.54	11.98
7	29.49	26.86	19	7.52	21.31	30.87	8.69	2.86	36.64	32.24	21.63	12.78
8	29.14	27.91	19.32	7.86	21.78	32.21	10.97	3.85	37.33	34.54	21.79	14.05

Table 3.6. Details of the Transplanted Spontaneously Developed Mammary Tumors collected from Female or Male Noble rats.

Noble Rat	Sex	Age	Tumor Location	Tumor Gross appearance	Histopathology
1	Female	14 months old	Right thoracic mammary gland	White and rubbery lump with plenty milk secretion	Benign secretory adenomna; Lactating alveoli with scanty connective tissues . (Figure 3.3.1)
2	Female	12 months old	Right thoracic mammary gland	White, rubbery, lump with milk secretion	Benign secretory fibroadenoma; Mixed stromal and epithelial pattern (Figure 3.3.2)
3	Male	13 months old	Right abdominal mammary gland	White, rubbery firm lump	Benign nonsecretory fibroadenoma; Concentric layers of connective tissue with few ductules (Figure 3.3.3)

Table 3.7 Immunohistochemical Analysis of Hormone Receptors in Normal and Neoplastic Mammary Tissues in Female Noble Rats

	Relative Immunoreactivity Intensity *				
	Estrogen Receptor α	Estrogen Receptor β	Progesterone Receptor	Androgen Receptor	Prolactin Receptor
Normal Mammary Gland	+++	++	++	+++	++
Spontaneously Developed:					
Benign Tumor	+++	++	+++	+++	+++
Malignant Carcinoma	+++	++	+++	+++	++
DMBA Induced Carcinoma	++	- / +	++	++	+++
T+E_2 Induced Carcinoma	+++	+++	++	+++	++
T+DES Induced Carcinoma	+++	+++	+++	+++	+++

Immunoreactivity intensity in arbitrary scales:
+++ Strongly Positive
++ Moderately Positive
+ Weakly Positive
- Negative

Chapter 4. Discussions

4.1 Comparison of the Incidence Rate of Spontaneously developed Mammary Tumors in Noble Rats with the Previously Reported Incidence Rate

In the present study, the incidence rate of spontaneous mammary tumors in Noble rats was found to be 45 % in the females and 3.13 % in the males. On the other hand, the incidence rate of the spontaneous mammary tumors in the aged Noble rats as reported by Dr RL Noble in two decades ago was 25 % in the females and 2 % in the males (Noble *et al,* 1975). In comparison, the incidence rate of spontaneous tumor in the male Noble rats of the present colony is higher than, but is still very close to, the reported figure. However, the incidence rate of the spontaneous mammary tumors in our colony of female Noble rats is much higher than the reported figure, it is nearly double the one reported by Dr RL Noble (Noble *et al,* 1975). Nevertheless, it is not uncommon that different incidence rates of spontaneous mammary tumors are reported in the same rat strain by different and even the same laboratories. For example, variability of incidence rates of spontaneous mammary tumors is also reported in the most commonly used rat strains, including the Sprague-Dawley (SD) and Wistar rats (Sher, 1972; Manik *et al.*, 1992; Kathleen & James, 1994). In the female SD rats, spontaneous tumor incidences of 55 %, 62 %, 64 % and even as high as 85 % were reported (Sher, 1972). The reported tumor incidences in male SD rats also range from 19 % to 44 % (Sher, 1972). The exact reasons for these variations are difficult to explain as a lot of factors can affect the spontaneous tumor incidence during the study. These include the difference in the habitat environments such as the dietary contents, the temporal length of dark/light cycle, background radiations and other factors such as the

presence of carcinogens or virus, the length of study, the age and mortality of the animals, the sample numbers, the germline mutations of the rat colony (Noble & Cutts, 1959; Sher, 1972). However, the involvement of virus can be excluded in this case. As females are more susceptible to the development of breast cancers, the effects of these possible factors are believed to be more significant in the females as compared with the males. All these possible factors may contribute to our present observation that there is great derivation of incidence rate of spontaneous mammary tumors in the female Noble rats but not in the males.

4.2 Comparison of the Incidence rate of Spontaneously Developed Mammary Tumors in Noble Rats with the Incidence Rate in Other Rat Strains

The incidence rate of spontaneous mammary tumors developed in Noble rats in the present study is 45 % in the females and 3.13 % in the males. These incidence rates are compared with the previously reported data in other extensively used rat strains. It is found that in many aged female rat strains, the spontaneous mammary tumors also occur with a frequency up to 45%. These include the Wistar (Sher, 1972; Walsh and Poteracki, 1994), F344 (Chandra & Frith, 1992), SD (Sher, 1972) and the Osborne-Mendel strains (Sher, 1972). On the other hand, the incidence rate of spontaneous mammary tumors in the male Noble rats is also similar to the previously reported data in the other rat strains, including the SD, F344 and Wistar strains (Sher, 1972; Noble *et al.,* 1975; Chandra *et al.,* 1992; Walsh & Poteracki, 1994).

4.3 Crucial Factors Influencing the Incidence Rate of Spontaneously Developed Mammary Tumors in Noble Rats

It is observed that the spontaneous mammary tumors develop much more commonly in the aged populations, normally when the Noble rats are over 1 year old. It is also observed that the female rats are much more susceptible to develop spontaneous mammary tumors as compared with the males. These observations are consistent with the previously reported data in the other rat strains, including the SD, F344 and Wistar strains (Noble & Cutts, 1959; Sher, 1972; Noble *et al.*, 1975; Chandra *et al.*, 1992; Walsh & Poteracki, 1994). The high incidence of spontaneously developed mammary tumors in the aged female population is possibly due to the lifetime accumulations of carcinogenic mutations in the aged animals and the high systemic estrogen levels in the female animals. Current views on the oncogenetic basis of female breast cancer suggest that the malignant transformation of the normal breast epithelium into an invasive carcinoma is a multistep process, in which a number of genetic alternations have occurred within various critical genes, that are directly or indirectly responsible for regulating certain important cellular processes, such as cell proliferation, differentiation, chromosomal replication and apoptosis (Lakhani, 1999). As temporal accumulations of these sporadic carcinogenic mutations are a prerequisite for the development of a tumor, breast cancer is thus more commonly found in the aged populations. On the other hand, the most well-documented risk factors of breast cancer, such as early menarche, late menopause and nulliparity, are associated with the most significant physiological changes in the estrogen secretion during a female's lifetime. These clinical observations strongly suggest that estrogen plays a crucial role in the development of human breast cancer (McPherson *et al.*, 2000). Therefore, the higher incidence of the

mammary tumors in the females is believed correlated to the higher endogenous secretions of estrogen. The possible mechanisms that estrogen acts as endogenous carcinogens in breast cancer will be discussed in the following sections.

4.4 Comparison of the T+E_2 Induced Mammary Tumors with the T+DES Induced Mammary Tumors in Female Noble Rats

In the hormone-induced mammary tumors of female Noble rats, the incidence rate in the testosterone and diethylstilbestrol (T+DES) treatment (55.55 %) is higher than that in the testosterone and 17β-estradiol (T+E_2) treatments (46.66 %). On the other hand, the average latency period of the T+ DES-induced mammary tumors (10 months) is also longer than that of the T+E_2-induced mammary tumors (8.86 months). Furthermore, the mortality of the T+DES-treated rats (55 %) is much higher than that of the T+E_2-treated group (25 %). These differences between the T+DES and T+E_2 treatments are probably due to the derivations in the estrogenic activity and carcinogenicity between the synthetic estrogen, DES, and the physiological occurring estrogen, 17β-estradiol. Both the DES and E_2 can bind to the estrogen receptors (ER) (Korach *et al.*, 1991). However, DES has a higher affinity for receptor binding as compared with E_2. As a result, DES leads to longer duration of ER activation and higher estrogenic activity in the affected animals (Marselos & Tomatis, 1992; 1992b). On the other hands, the biological effects of these estrogenic compounds on the mammary gland may also result indirectly from their metabolic activation to reactive metabolites (Russo *et al.*, 2001). The resulted systemically active metabolites may possess different biological activities and finally lead to the disparate behaviors between the T+DES and T+ E_2-induced mammary tumors. Previous studies had already shown that both the DES and E_2 were able to induce

lobuloalveolar hyperplasia or mammary tumor in Noble rats (Colerangle & Roy, 1995; Liao *et al.*, 1998; Odum *et al.*, 1999; Xie *et al.*, 1999) or in other rat strains such as the ACI rats (Dunning *et al.*, 1949; Schull *et al*, 1997; Harvell *et al.*, 1999). In the present study, it is found that DES is able to provide higher incidence rate of induced-mammary tumors in female Noble rats as compared with E_2. However, higher frequency of mortality and abnormality in the reproductive organs, such as ovary and uterus, is also resulted in the T+DES-treated animals. It has already been established that DES is a carcinogen and its carcinogenicity has been demonstrated in rats, mice, hamsters, dogs, monkeys and even human (Marselos & Tomatis, 1992; 1992b). The target organs of DES include the vagina, cervix, uterus, ovary, and the mammary gland (Marselos & Tomatis, 1992; 1992b). Hence, the high death rate and the frequent abnormalities induced in ovary and uterus observed in the T+DES-treated Noble rats may be due to the strong carcinogenicity of DES.

4.5 Comparison of the Incidence Rate & Latency Period of the Hormone Induced Mammary Tumors in Noble Rats with the Previously Reported Data

In a previous study by Dr YC Wong and colleagues, it was reported that a combined treatment of androgen (testosterone propionate) and estrogen (17β-estradiol benzoate) could induce a high incidence of mammary tumors in female Noble rats within relatively short time (Xie *et al.*, 1999). The incidence rate of the induced-mammary tumors was 52.78 %, with average tumor latency period of 5.82 months. In our study, the incidence rate of the $T+E_2$ induced mammary tumor is 46.66 % and the tumor latency period is 8.86 months. In comparison, the incidence rate of the $T+E_2$-induced mammary tumors under the present study (46.66 %) is lower than, but is still similar to, the figure reported by Dr. YC Wong (52.78 %).

However, the tumor latency period of the present study (8.86 months) is much longer than the reported latency period (5.82 months). These differences may be due to the variations in the dosage of testosterone administered. In the present study, only two 2-cm testosterone tubings were implanted subcutaneously into the female Noble rats, as compared with four 2-cm testosterone tubings in Dr YC Wong's study. These observations suggest that the role of androgen in this hormone-induced model of female Noble rats is to speed up the estrogen-induced mammary carcinogensis. This result is consistent to the hypothesis proposed by Dr YC Wong (Xie *et al.*, 1999b).

4.6 Comparison of the Phenotypic Behaviors in Spontaneously Developed Mammary Tumors with the Hormone Induced Mammary Tumors in Female Noble Rats

In comparison, the incidence rate of the spontaneously developed mammary tumors (45 %) is similar to that of the T+E_2-induced tumors (46.66 %). However, the latency period of the hormone-induced tumors is much shorter than that of the spontaneously occurring neoplasms. The average age of the female Noble rats for the occurrence of spontaneous mammary tumor is about 14 months old. However, the average age of the female Noble rat for the incidence of hormone-induced mammary tumors is much lower and is only 10 months old. These observations suggest that the hormonal treatment is able to speed up the carcinogenesis without altering the final tumor incidence.

It has been repeatedly shown that estrogen is a mitogen and is able to stimulate cell cycle progression in the normal mammary epithelium and the ERα expressing cancer cells, thereby resulting in the proliferation of the normal and neoplastic mammary tissues (Foidart *et al.*, 1998; Zhou *et al.*, 2000). The high

incidence rate of the spontaneously developed mammary tumors in female Noble rat suggests that sporadic oncogene mutations may occur frequently in this rat colony. Sporadic oncogene mutations are not uncommon in rat strains. It was previously reported that spontaneous oncogenic GGA to GAA mutations in the 12th codon of the *Hras* gene are frequently detected during the normal development of the mammary epithelium of female F344 rats (Cha *et al.*, 1994). On the other hand, it is now clear that proliferating mammary cells are prerequisites for the breast carcinogenesis (Nandi *et al.*, 1995). Therefore, estrogen may contribute to the initiation of the mammary gland carcinogenesis by increasing the rate of cell proliferation in the breast epithelium, thereby enhancing the occurrence of oncogenic mutation in the mammary glands. The whole mount preparation of the hormone-treated mammary glands have demonstrated that sex steroids are potent mitogen in the mammary glands of female Noble rats as extensive lobuloalveolar proliferation and differentiation are observed only ten days post the hormonal treatment. Besides, it is also suggested that estrogen can initiate mammary gland carcinogenesis directly by metabolic activation to form DNA adducts. The resulting mutations may leads to the development of breast tumors (Cavalieri *et al.*, 1997; Liehr, 1997).

A comparison between the histopathology of the spontaneously developed mammary tumors and the hormone-induced mammary tumors reveals that the hormone-induced tumors are much more malignant than the spontaneous tumors. Most of the mammary tumors develop spontaneously are benign fibroadenomas whereas those induced by hormones are poorly differentiated carcinomas. On the other hand, it is known that the mammary gland carcinogensis is a multistage process (Lakhani, 1999). The transition of the mammary gland from normal epithelium to invasive carcinoma involves a lot of intermediate pathological stages

such as non-atypical hyperplasia, atypical hyperplasia and *in situ* carcinoma. Based on our observations and the information from the literatures, it is hypothesized that the role of estrogen in the hormone-induced model of female Noble rats is to initiate the neoplastic transformation in the mammary gland. On the other hand, androgen may act as a promoter to speed up the development of the breast tumors. As more malignant carcinoma is observed in the hormone-induced model as compared with the spontaneously developed model, it is supposed that the promotion effect of androgen, alone or with estrogen, in the carcinogenesis is mainly on the malignant transformation of the benign tumors.

4.7 Comparison of the Behaviors of Carcinogen Induced Mammary Tumors with Spontaneously Developed & Hormone Induced Mammary Tumors in Female Noble Rats

The incidence rate of the carcinogen-induced mammary tumors (80 %) is much higher than that of the spontaneously developed (45 %) and hormone-induced mammary tumors (T+DES: 55.55 %; T+E_2: 46.66 %) in female Noble rats. The mean latency period for the development of the carcinogen-induced mammary tumor is also much shorter (6.38 months) than that of the spontaneously occurring and the hormone-induced neoplasms. These disparate observations may suggest that the spontaneously developed and hormone-induced mammary tumors arise through a different carcinogenic mechanism as compared with the carcinogen-induced neoplasms in female Noble rats. This interpretation is further supported by the observations that the hormone receptor expression patterns in the carcinogen-induced mammary tumors are different from that of the spontaneously developed and hormone-induced mammary tumors (to be discussed later). Pieces of evidence

can also be obtained from the literatures. It is well established that the biological effects of the steroid hormone, testosterone and 17β-estradiol, are mainly mediated by binding with specific cytoplasmic receptors. The activated hormone-receptor complex then migrates to the nucleus, complements to the hormone-responsive genes and finally modulates transcription of the target genes (Russo *et al,* 1999; Hansen & Bissell, 2000). In the hormone-induced models, a prolonged and continuous supply of the exogenous hormone results in a consistent activation of the hormone receptors. The subsequent unregulated transcriptions of the corresponding hormone responsive genes are believed related to the mammary gland carcinogenicity. This may provide the basis of the hormone-induced carcinogensis in female Noble rats. For the spontaneously developed models, the sporadic mutations may result in unbalanced secretions of the steroid hormones or an unregulated activity of the hormone receptors. The unregulated transcriptions of the corresponding hormone responsive genes then induce the development of mammary tumors. Therefore, it is hypothesized that both the hormone-induced and spontaneously developed mammary tumors arise through a similar carcinogenic mechanism. On the other hand, although the chemical structure of DMBA is very similar to the estradiol, this chemical requires metabolic activation for its mammary gland carcinogenicity (Clarke, 1997). Liver and mammary glands are the important sites for the activation of DMBA. The ultimate carcinogenic DMBA metabolites are systemically active. It can bind to specific genes in the breast and leads to the development of mammary tumors (Singletary, 1990). The target genes for the DMBA may be in different loci as compared with the hormone responsive genes and this may leads to the derivations in the carcinogenic mechanisms in the mammary glands. Due to this discrepancy, the incidence rate, latency period and behaviors of

the carcinogen-induced mammary tumors are different from that of the hormone-induced and spontaneously developed mammary tumors.

4.8 Comparison of Expression Patterns of Hormone Receptor Proteins in Spontaneously Developed, Hormone Induced & Carcinogen Induced Mammary Tumors in Female Noble Rats

The expression patterns of hormone receptors as detected by immunohistochemistry in the normal and neoplastic mammary tissue are summarized in Table 3.7, in which the intensities of immunoreactivity are expressed in arbitrary values. It has been observed that the hormone receptor statues in the spontaneously developed benign and malignant mammary tumors are very similar to those in the T+E_2 and T+DES-induced tumors. In both the spontaneously developed and the hormone-induced models, the mammary tumors exhibit moderate to strong expression of different hormone receptors, including both the steroid receptors (ERα, ERβ, PR and AR) and the cytokine receptor (PRLR). In contrast, the carcinogen-induced neoplasms only exhibit moderate immunoreactivity for the steroid receptor (ERα, ERβ, PR and AR). However, a very strong immunostaining for PRLR was detected in these mammary tumors. These observations demonstrate additional pieces of evidence supporting the notion that the process of mammary gland carcinogenesis in the carcinogen-induced model may be different from that of the spontaneously developed and hormone-induced models in female Noble rats. As mutations of different oncogenes and tumor suppressor genes are involved during the mammary gland carcinogenesis in these different animal models, the conversion of the varied genetic information into a defined phenotype may lead to a discrepancy in the hormone receptor status between the carcinogen-induced mammary tumors and

the spontaneously developed as well as the hormone-induced neoplasms. The expression of individual hormone receptor in the spontaneously develop, hormone-induced and carcinogen-induced mammary tumors in female Noble rats are further discussed as follows.

4.9 Expressions of ERα & ERβ Proteins in Spontaneously Developed, Hormone Induced and Carcinogen Induced Mammary Tumors in Female Noble Rats

Moderate to strong nuclear ERα immunoreactivity was detected in the spontaneously developed, hormone-induced and carcinogen-induced mammary tumors. On the other hand, the ductal and alveolar epithelial cells in the normal mammary glands also exhibited strong ERα nuclear expressions. These nuclear immunostaining for ERα suggests that the receptors have been activated by its ligand and is functionally active for modulation on ER-responsive gene transcriptions in both the normal and neoplastic mammary tissues. The uniform and intense expressions of the functionally active ERα clearly indicate an obligatory role for estrogen in the developments and functions of the normal mammary glands as well as the inductions and progressions of the mammary tumors in female Noble rats (Russo & Russo, 1997).

Native ERα is an approximately 67kDa protein identified in the rodent uterus (Jensen & Jacobson, 1962; Horigome *et al.,* 1987). Western blot analysis in previous studies has shown that ERα could exist in multiple isoforms (Geffroy-Roisne *et al.,* 1993; Faye *et al.,* 1996). The existence of these ERα isoforms has been attributed to the proteolytic cleavage and phosphorylation of the receptor protein or possible association of the receptor with other carrier proteins. A recent study has shown that several ERα isoforms are involved in the hormone-induced mammary gland

carcinogenesis in male Noble rats (Liao *et al.,* 1998). In the present study, it was found that a 67kDa native protein was expressed in all form of mammary tumors. In comparison, it has been shown that the DMBA-induced neoplasms exhibited a weaker expression of this native 67kDa ERα protein. On the other hand, some of the DMBA-induced neoplastic samples also exhibit a weak expression of the putative 50 kDa ERα isoforms. Moreover, the putative 50 and 54 kDa ERα isoforms were also detected in the spontaneously developed benign tumors and malignant carcinomas. Although the functional significances of these ERα isoforms are unknown, their presence suggests the possibility that the signaling mechanisms of ERα may vary between the models. A relatively intense ERα immunoreactivity as detected in the spontaneously developed and hormone-induced mammary tumors indicates that the developments of these tumors are relatively more dependent on estrogen, as compared with the carcinogen-induced mammary tumors in female Noble rats. Additional support for the greater estrogen dependency of the spontaneous and hormone-induced mammary tumors is demonstrated by the ERβ status in these tumors.

In the normal mammary glands of female Noble rats, a moderate ERβ immunoreactivity was detected in most of the ductal and alveolar epithelial cells. Similar observation on ERβ status was previously described in both human breasts and rodent mammary glands. ERβ expression was detected in both normal and neoplastic human breast tissues (Warner *et al.,* 2000). In rats, it was also reported that a large proportion of mammary epithelial cell expressed ERβ proteins at all phrases of breast development and endocrine stimulations, including puberty, pregnancy and lactation (Saji *et al.,* 2000). In the present study, expression of ERβ proteins in the rat mammary tumors was found variable between different animal

models. Moderate to strong ERβ expressions were detected in both spontaneously developed and hormone-induced mammary tumors. However, in the carcinogen-induced breast tumors, only negative to weak ERβ immunoreactivity was observed. Since the discovery of ERβ from rat ovary and prostate in 1996 (Kuiper *et al.*, 1996), the biological roles of ERβ and its interaction with ERα in the mammary glands are still undefined. The positive expression of ERβ in mammary tumors shows that this steroid receptor also plays a role in the mammary gland carcinogenesis. The intense co-expressions of ERα and ERβ in the spontaneously developed and hormone-induced mammary tumors in female Noble rats also indicate that there could be some degree of interactions between the two ER isoforms. On the other hands, this observation also suggests that the development of these mammary tumors may be dependent on estrogen to larger extent as compared with the carcinogen-induced neoplasms. In the present study, the observed differences in the behaviors and hormone receptor status between the carcinogen-induced mammary tumors and the spontaneously developed as well as the hormone-induced neoplasms are interpreted as a result of the discrepancy in the process of carcinogenesis. As mutations of different oncogenes and tumor suppressor genes are involved during the mammary gland carcinogenesis in these different animal models, the conversion of the varied genetic information into a defined phenotype may lead to a discrepancy in the hormone receptor status between the carcinogen-induced mammary tumors and the spontaneously developed as well as the hormone-induced neoplasms.

4.10 Expressions of PR Proteins in Spontaneously Developed, Hormone Induced and Carcinogen Induced Mammary Tumors in Female Noble Rats

The expression patterns of PR in the mammary glands and breast tumors of female Noble rats are similar to that of the ERα. In the normal mammary gland and all different classes of mammary tumors, a moderate to strong PR immunoreactivity was observed. The co-expression of ERα and PR in the normal and neoplastic mammary epithelium is not unique in Noble rats. Previous studies on human breast cancer have shown that about 50% of ERα-positive breast tumors are also positive for PR (Sharyl & Nancy, 1999; Bamberger *et al.,* 2000). A similar observation is also described in the carcinogen-induced mammary tumors in rodent, including both DMBA-induced (Iino *et al.,* 1990) and NMU-induced (Martin et al., 1996) neoplasms. In the NMU-induced breast tumors, it is reported that all the induced neoplasm are positive for both ERα and PR regardless the tumor histopathological varieties (Martin et al., 1996). On the other hand, co-expression of ERα and PR has also been depicted in the normal epithelium in human breast tissues and rodent mammary glands (Russo *et al.,* 1999). These extensive co-expressions of ERα and PR in both normal and neoplastic breast tissues can be explained by the fact that ERα is a key transcription factor for the expression of PR and induction of PR expression is one of the main functions of ERα in the breast epithelium (Read *et al.,* 1988; Shigehira *et al.,* 2000). Therefore, the expression of PR in ERα-positive mammary tumors is an expression of ERα functionality (Martin *et al.*, 1996). In the present study, there is intense co-expression of ERα and PR in the normal mammary glands as well as the spontaneously developed, hormone-induced and carcinogen-induced mammary tumors in female Noble rats. The expression of PR may be

induced by the functionally active ERα as these mammary glands and tumors are also positively stained with ERα.

Western blot analysis revealed that mammary glands and all classes of mammary tumors in female Noble rats express PR-A2 isoforms. Elevated expressions of PR-B and PR-A1 isoforms are also detected in the spontaneously developed carcinoma. Due to the inadequacy in the understanding of biological functions of PR isoforms, the significance of these observations is still undefined in the present stage. On the other hand, there are also expressions of PR-C isoform in the normal mammary glands, spontaneously developed, carcinogen-induced and T+E_2-induced mammary tumors. However, in the T+DES induce carcinomas, protein expression of PR-C was not detected. It is speculated that the difference in the PR-C expression between the T+DES-induced carcinomas and other classes of mammary tumors may be due to the distinct estrogenic activity of the synthetic estrogen, DES, as compared to the physiological estrogen, E_2. In the T+DES-treated animals, DES may induce a distinct estrogenic regulation on PR expressions. Therefore, absence of PR-C isoform is only observed in the T+DES-induced carcinomas, but not in the other classes of mammary neoplasms and the normal glands.

4.11 Expressions of AR Proteins in Spontaneously Developed, Hormone Induced and Carcinogen Induced Mammary Tumors in Female Noble Rats

Positive AR immunoreactivity was detected in the normal mammary glands of female Noble rats. The co-expression of AR and ER (ERα & ERβ) in the normal mammary epithelium could probably explain the high sensitivity of female Noble rats to the combined androgen and estrogen treatment for the induction of mammary

tumors (Xie *et al.,* 1999). On the other hand, a moderate to strong AR reactivity was also detected in the spontaneously developed, hormone-induced and carcinogen-induced mammary tumors. These consistent expressions of AR immunoreactivity in all classes of mammary neoplasms suggest that androgen may play a role in the mammary gland carcinogensis. Western blot analysis also shows that the AR-B isoform was markedly elevated to different extents in all classes of tumors. In the spontaneously developed mammary tumors, an elevated expression of the AR-A protein was also detected. Although the biosynthesis and function of the androgen receptor isoforms in the mammary gland and tumors are largely unknown in the current stage, elevated expressions of the AR isoforms in the neoplastic mammary tissues suggest the possibility that androgen and its receptor may participate in the mammary gland carcinogensis (Catalano *et al.,* 2000). A review on literatures suggests that the role of androgen on the development of the breast tumor can be multi-discipline. On one hand, androgen can promote the proliferation of breast cancer cells directly via the androgen receptor-mediated mechanism or by its stimulation on the synthesis of other growth factors (Xie *et al,* 1999b; 1999c; 2000). On the other hand, the action of androgen may also be indirect. Firstly, androgen can be converted into estrogen by the enzyme aromatase (Bernstein and Ross, 1993). Clinical studies have already shown that estrogen, derived from the systemic conversion in peripheral tissues or from the local conversion in the breast tumors, participates in the maintenance and growth of the breast tumors in the carriers, especially in the postmenopausal females (Chen, 1998). Alternatively, androgen can also act indirectly by increasing the circulating amount of free estradiol, via either reducing the secretions of hepatic estradiol-bound sex hormone binding globulin

(SHBG) by the liver or decreasing the fraction of the SHBG, thereby providing substantial amount of estrogen for tumor growth (Lonning *et al.,* 1995).

The expressions of both AR-A and AR-B isoforms in the spontaneously developed mammary tumors provide an interpretation for the continuous growth of the spontaneous mammary tumors in the bilateral ovariectomized Noble rats. The presence of the steroid hormone receptors (ERα, ERβ, PR and AR) and cytokine receptor (PRLR) (to be discussed later) in the spontaneously developed mammary tumors suggests that the growth and development of the spontaneous neoplasm, to large extents, depends on the steroids (estrogen, progesterone and androgen) and peptide hormones (prolactin). Although ovariectomy is able to reduce the endogenous secretions of estrogen and progesterone, the growth of spontaneously mammary tumors is still under the influences of androgen and prolactin. The alterations in the hormonal environments following ovariectomy may modify the hormone dependency of the spontaneously developed mammary tumors. After the ovariectomy, androgen and prolactin, or with other endocrine factors, may be responsible for the growth of the spontaneous mammary neoplasms. Therefore, the mammary tumors continue to grow even the endogenous secretions of estrogen and progesterone is significantly reduced.

The intense expression of AR in the hormone-induced mammary tumors in female Noble rat is consistent with a previous reported (Xie *et al.,* 1999b). In a series of studies carried out by Dr YC Wong and his associate in the hormone-induced models in female Noble rat, the promoting role of androgen in the mammary gland carcinogenesis is speculated (Xie *et al,* 1999b; 1999c; 2000). It is suggested that androgen acts by upregulating the Bax protein expression and thus inducing a high apoptotic activities in the normal mammary epithelial cells. This results in a selective

pressure favoring the clonal expression of the neoplastic cells. On the other hand, it is also speculated that androgen also provokes the selective mechanism via an upregulation of TGF-β1 expression. TGF-β1 has the ability to inhibit the growth of normal epithelial cells. When the neoplastic cells become refractory to the biological actions of TGF-β1, the presence of TGF-β1 will pose a selective advantage for the outgrowth of the transformed clone because it only inhibits the proliferation of the normal cells. Thus, the final outcome of androgen's effects would be an increased mammary tumor incidence and shorter latency period of mammary tumors.

A strong AR Immunoreactivity was also detected in the carcinogen-induced mammary tumors in female Noble rat. Previous *in vitro* and *in vivo* studies also reported that AR expresses in the DMBA-induced mammary tumors (Ip *et al.*, 1978; Vignon *et al.*, 1979). In contrast to the stimulating role of androgen in the hormone-induced mammary gland carcinogenesis, it has been shown that androgen and AR are involved in the inhibition of growth of the DMBA-induced mammary tumors (Gatto *et al.*, 1998).

So far, the roles of androgen and AR in the mammary gland carcinogenesis are still controversial. In the present study, it is speculated the continuous growth of the spontaneously developed mammary tumors in the ovariectomized Noble rats and the shortened latency period of the hormone-induced mammary tumors are due to the stimulatory role of androgen and AR on the carcinogenic process. However, in the DMBA-induced models, it is previously report that androgen and AR are involved in the regression of the breast neoplasms (Gatto *et al.*, 1998). These conflicting observations may suggest the possibility that androgen and AR plays a different role in the development of breast cancer between the carcinogen-induced models and the spontaneously developed as well as the hormone-induced models.

This discrepancy in the biological activities of androgen and AR may be a result of the difference in the carcinogenic mechanisms between these models. However, more studies are required to confirm this hypothesis.

4.12 Expressions of PRLR Proteins in Spontaneously Developed, Hormone Induced and Carcinogen Induced Mammary Tumors in Female Noble Rats

A moderate to strong PRLR immunoreactivity was detected in both normal mammary glands and breast tumors in the spontaneously developed, hormone-induced and carcinogen-induced models of female Noble rats. These observations are consistent with the clinical studies on human breast cancers. It was previously demonstrated by immunocytochemistry that over 93 % of normal human breast tissues and over 95 % of the human breast tumor expressed the PRLR (Reynolds *et al.*, 1997). The involvement of prolactin and PRLR are also well studied in the murine and rodent mammary cancers. In mice, prolactin is found directly contributing to the etiology of both spontaneously developed and carcinogen-induced mammary carcinomas (Welsch and Nagasawa, 1977). Prolactin can also interact with the ovarian steroids synergistically in the promotion on the growth of human breast cancer xenografts in nude mice (Leung and Shiu, 1981). In rats, there is a direct association between the serum prolactin level and the susceptibility of the animals to the chemical induction of mammary tumors (Boyns *et al.*, 1973). Tumors induced in rat by NMU or DMBA are also dependent on prolactin for sustained growth (Mershon *et al.*, 1995). In the present study, the presence of PRLR in mammary tumors of female Noble rats further suggests that prolactin participates in the mammary gland carcinogensis.

On the other hand, in the DMBA-induced mammary tumors, it is observed that the immunoreactivity of PRLR is stronger than the reactivity demonstrated by the steroid hormone receptors, including ERα, ERβ, PR and AR (Table 3.7.). These observations further solidify the participation of prolactin in the carcinogen-induced model. The strong PRLR reactivity suggests that the growth of the DMBA-induced tumors could be largely depend on prolactin. This observation is consistent with the previously reported data (Mershon *et al.*, 1995).

The expression of PRLR in the spontaneously developed mammary tumors in female Noble rat also provides a reasonable explanation for the continuous growth of the spontaneous neoplasm in the ovariectomized Noble rats. The strong PRLR reactivity suggests that the growth of the spontaneous neoplasm may be also dependent on prolactin. After the depletion of progesterone and estrogen by ovariectomy, prolactin, alone or in combined action with other hormonal factors, such as androgen, is responsible for the sustained growth and development of the breast tumors.

In the present study, it is observed that the expression patterns of ERα and PR in the mammary tumors of female Noble rats are very similar to that of PRLR. Breast tumors in the spontaneously developed, hormone-induced and carcinogen-induced models of female Noble rats, all exhibit moderate to strong PRLR, ERα and PR immunoreactivities. These observations suggest that the expression of PRLR is correlated with the expression of ERα and PR. The co-expression of PRLR with steroid receptor is also depicted in the human breast tumors (Bonneterre *et al.*, 1982; Bonneterre *et al.*, 1986). These observations also raise a possibility that the sex steroid hormones, estrogen and progesterone, may interact synergistically with prolactin to control the neoplastic growth of the mammary gland. In the current

stage, the cellular mechanism for this hormonal synergy is still uncertain. However, according to our observation, the synergy mechanism may involve the cellular co-expression of steroid and prolactin receptors and their cross-regulations.

The commercial available antibody used in the immunohistochemistry and Western blot analysis under the present study is able to recognize the ligand binding site of the rat PRLR. This antibody is also capable of inhibiting the interaction of prolactin with PRLR *in vitro* (Okamura *et al.*, 1989). As the long and short isoforms of the PRLR only differ in the length of the cytoplasmic domain (Shirota *et al.*, 1990), this PRLR antibody can also recognize both the short and long form of PRLR. It has been reported that PRLR is widely expressed in different tissues, among which the mammary gland expresses the long form predominantly whereas the liver expresses the short form predominantly (Nagano & Kelly, 1994). Due to the enzymatic modifications during or after the translation and the difference in tissue and species source, immunoreactive PRLR varying in size from ~40 kDa to 100 kDa are depicted (Guillaumot and Cohen, 1994). In the present study, western blot analysis for PRLR has detected expressions of a PRLR protein with a molecular size of about 65kDa in the normal and neoplastic mammary samples. This protein is regarded as the long isoform PRLR. The physiological roles of the long form PRLR in lactating mammary gland are well established. It is reported that long PRLR is involved in the stimulation of milk protein synthesis at both transcriptional and translational levels (Lesueur *et al.*, 1991; Shirota *et al.*, 1995). The presence of long PRLR in the mammary tumors of female Noble rats further suggests that this PRLR isoform also participates in the mammary gland carcinogenesis.

Chapter 5. Conclusions

In the present study, it was observed that female Noble rats were susceptible to carcinogen and steroid hormone treatments for the induction of mammary tumors. On the other hand, spontaneous mammary tumors were also developed in high incidence rate in female Noble rats. A comparison of the incidence rate, lengths of latency period and histopathology of the mammary tumors developed in these three different rat models suggests that hormonal treatment is able to accelerate the spontaneously developed carcinogenesis in the mammary glands of female Noble rats. On the other hand, it is believed the carcinogen-induced mammary gland carcinogensis occurs through a different mechanism as compared with the spontaneously developed and hormone-induced mammary gland carcinogenesis. The differences of the hormone receptor status between the carcinogen-induced mammary tumors and spontaneously developed as well as hormone-induced tumors as revealed by immunohistochemistry and western blotting further support the notion that the process of mammary gland carcinogenesis in the carcinogen-induced model is different from that of the spontaneously developed and hormone-induced models in the female Noble rats.

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